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Validation Of Complete Blood Count Methodology, And Determination Of The Relationship Between Endoparasite Load And Erythrocyte Values In New World Camelids

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To the Graduate Council:

I am submitting herewith a thesis written by Lisa C. Viesselmann entitled "Validation Of Complete Blood Count Methodology, And Determination Of The Relationship Between Endoparasite Load And Erythrocyte Values In New World Camelids." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Comparative and Experimental Medicine.

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Validation Of Complete Blood Count Methodology, And Determination Of The Relationship Between Endoparasite Load And Erythrocyte Values In New World Camelids

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ABSTRACT

Background: Accurate measurement of RBCs (red blood cells) by automated hematology analyzers such as the ADVIA 120/2120 requires isovolumetric cell sphering; however, camelid RBC membranes are resistant to shape change. There are no published reports of method validation for hematologic analysis of camelid blood. *Mycoplasma haemolamae* and gastrointestinal nematodes can cause anemia in camelids. Parasite control programs aim to suppress parasite loads without promoting resistance, but there are few evidence-based guidelines for acceptable parasite loads in camelids.

Objectives: 1) Demonstrate whether camelid RBCs sphere in the ADVIA sphering reagent, and determine the optimal ADVIA setting for CBC (complete blood count) analysis, 2) Compare *M. haemolamae* PCR status with RBC values, and 3) Determine the fecal egg count (FEC) threshold above which RBC values are consistently below the median of the reference interval.

Methods: Camelid and canine blood were each added to ADVIA sphering reagent or saline, and evaluated by light microscopy for erythrocyte sphering. Camelid blood was analyzed on an ADVIA 120 hematology analyzer using one of three species settings, and values compared to manual measurements, including packed cell volume (PCV), Z2 Coulter counter RBC count, and calculations of other RBC values. *Mycoplasma haemolamae* was detected by real-time PCR. The number of trichostrongyle eggs per gram (epg) of feces was determined using the Modified McMaster's test.

Results and Conclusions: Camelid erythrocytes do not sphere when mixed with ADVIA sphering reagent. The ADVIA 120 equine setting provides the closest approximations to Z2 counter RBC count estimates, but ADVIA results for most other RBC values appear inaccurate. PCV,

hemoglobin, and RBC count are not significantly different between *M. haemolamae* positive and negative animals, but are significantly lower in animals with FEC> [greater than] 600epg. For all animals with FEC>600epg, RBC values are below the medians of the reference intervals. Positive *M. haemolamae* PCR is not associated with lower RBC values in healthy camelids, consistent with previous reports that most infections are subclinical. Maintaining FEC below 600 epg is recommended in camelids.

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INTRODUCTION

Camelid RBC structure and function

Overview

Camelid species, including the camel (*Camelus dromedarius* and *C. bactrianus*), llama (*Lama glama*), alpaca (*Vicugna pacos*), vicuña (*Vicugna vicugna*), and guanaco (*Lama guanicoe*), have erythrocytes that are unique in shape, size, and biochemical composition compared to those of other mammalian species, including humans. In contrast to the biconcave disc shaped RBCs of most mammalian species, camelid RBCs are elliptical and flat, and have a smaller volume than those of many other animals.[1-4] Additionally, camelid RBCs are rigid, less deformable, and less susceptible to osmotic lysis than those of other mammals.[2,4,5] Deformability, or the ability of a cell to change its shape without breaking, is typically considered a beneficial feature for RBCs as they flow through vessels of varying size (large arteries to small capillaries), and are subjected to shearing stresses in flowing blood.[6] In human patients with hereditary elliptocytosis as a result of various RBC membrane protein deficiencies, a diagnostic hallmark of the disease is RBC fragmentation secondary to decreased deformability.[7] A report of canine elliptocytosis also documented both decreased deformability and decreased mechanical stability of RBC membranes in the proband compared to a healthy control dog, although RBC fragmentation was not sufficient to cause clinically significant hemolysis in this case.[8] However, the flat, elliptical shape and rigidity of the camelid RBC appear to offer physiologic benefits for these animals, likely by providing increased effective surface area for gas exchange (important in high altitude environments with low oxygen tension), and by increasing the cells' resistance to osmotic lysis (important in arid environments where water is rapidly consumed in large volumes when it is found).[4] The potential problem of fragmentation as an elliptical, relatively non-deformable cell

passes through small capillary beds appears to be averted in these species as a result of their small cell size.[4]

Studies of camelid RBCs

Camelid RBCs have been the subject of intensive study by both clinical and basic scientists due to their unique physical and biochemical properties. The elliptical shape of camelid RBCs on stained blood films was first described in 1875,[9] and approximately 100 years later the scanning electron microscope (SEM) provided a three-dimensional image of these cells, which multiple authors have described as "thin and wafer-like."[10,11] SEM images of both camel and llama erythrocytes mixed with human erythrocytes showed that camelid RBCs are both smaller and flatter than human RBCs.[12] The author of the same study also concluded that camelid RBCs have a higher concentration of hemoglobin within their smaller volumes, based on a higher relative mean corpuscular hemoglobin concentration (MCHC) compared to that of human RBCs analyzed with the same method.[12] This finding has also been described in more recent reviews of camelid hematology.[2] In 2005, Bogner, et al. demonstrated that camel erythrocytes have similar water to protein dry mass ratio as dehydrated human erythrocytes.[13] Given that water-protein interactions increase the osmotically non-responsive fraction of water within the cell, an increased protein (hemoglobin) concentration within camelid erythrocytes may contribute to the decreased rate of diffusion of water out of the cells, consequently increasing their resistance to dehydration relative to human erythrocytes.[13] The same group of researchers suggested that although the concentration of water in camel and llama erythrocytes is lower than that of normal human RBCs, the osmotically non-responsive (protein-bound) fraction of water is actually higher, possibly due to increased numbers of charged amino acid residues on their hemoglobin molecules, increasing the hydrophilicity of camelid hemoglobin.[14] The combination

of increased hemoglobin water affinity and cellular hemoglobin concentration in camelid RBCs may therefore reflect an adaptive resistance to osmotic dehydration in arid environments.[13,14] The high water-binding capacity of camelid RBCs may also make them more resistant to over-hydration, as might occur after rapid consumption of a large volume of water following a period of dehydration. Camel RBCs have been shown to expand their volume up to 196% with exposure to hypotonic saline prior to lysing (low osmotic fragility), despite the general trend of increasing osmotic resistance with increasing cell volume among other domestic animals.[15] In an SEM study of camel RBCs following experimental dehydration and rehydration, minimal changes were observed in RBC size and shape, and manual hematocrit and refractometric plasma protein measurements changed by less than 5% over the course of the study, suggesting a mechanism of rapid equilibration of water between intracellular and extracellular compartments.[16]

In addition to intracellular differences in hemoglobin structure and concentration, camelids also exhibit differences in their RBC membrane structure and protein composition compared to other domestic mammal species. While camel RBC membranes have similar lipid composition relative to those of humans and other mammals, the overall ratio of proteins to lipids in the RBC membranes is increased (approximately 3:1).[17] Additionally, while camel RBCs appear to have similar membrane proteins as those described in humans, the distribution and relative proportions of these proteins differ between the species. Gel electrophoresis studies of extracted membrane proteins show that camel RBCs have identifiable bands comparable to human peripheral membrane proteins (spectrins) and integral membrane proteins (band 3, band 5, etc). However, the peripheral membrane protein bands are smaller and the integral

membrane protein bands (especially band 3) are more distinct and appear to predominate.[17] This study also demonstrated that the integral membrane proteins are more closely organized in the camel RBC membrane, compared to the peripheral membrane proteins which have a similar concentration per unit of surface area as those in the human RBC membrane. These authors suggested that the interactions of these integral membrane proteins contribute to increased mechanical stability of camel RBCs (observed in response to sonication). Furthermore, reversibility of these protein interactions might allow for the dynamic swelling (vs. lysis) of the membrane that has been observed in response to plasma hypotonicity.[15,17] Mechanical stability has also been demonstrated in the form of decreased deformability of camel RBCs when exposed to fluid shear stresses. In contrast to the erythrocytes of most other domestic mammal species, which deformed readily under shear stress, camel (and llama) erythrocytes did not deform but became oriented in the direction of the fluid flow.[18]

Additional work with camel and alpaca erythrocytes suggest that RBC membrane stability in these species is at least partially due to interactions between band 3 and peripheral membrane proteins such as ankyrin and protein 4.1, as removal of these proteins as well as the cytoplasmic portion of band 3 resulted in increased rotational mobility of band 3 (decreased structural organization within the membrane).[19] Studies using llama erythrocytes have resulted in similar findings, namely that there is a 2.5-3x higher concentration of band 3 in camelid RBC membranes compared to human RBCs as a consequence of decreased relative surface area between the species (the absolute number of band 3 proteins is similar).[20] The mechanical benefit of this higher concentration of integral membrane proteins seems appropriate, however

the physiologic significance of having an increased concentration of this anion transport protein is uncertain.[20]

Camelid erythrocytes have decreased water content, increased resistance to osmotic and mechanical lysis, and decreased deformability, features which are important for life in arid environments. However, these animals are also adapted to high altitude environments as a result of increased oxygen affinity of their RBCs relative to other mammalian species. This high RBC oxygen affinity has been documented in all camelid species (wild and domestic), regardless of whether the animals are currently living at high altitudes, suggesting that it is an intrinsic characteristic of the Camelidae family. Increased oxygen affinity in this family appears to be secondary to amino acid substitutions in hemoglobin that limit its binding to 2,3-diphosphoglycerate (2,3-DPG), the primary molecule responsible for decreasing in vivo hemoglobin oxygen affinity.[21,22] Adult alpacas also retain higher proportions of fetal hemoglobin molecules than adult animals of other species, which have higher affinity for oxygen than adult hemoglobin molecules.[4]

Automated hematology analysis as it relates to camelid RBCs

Several of the unique structural features of camelid RBCs described above contribute to adaptation to life in arid and/or high altitude environments, however these same features also pose challenges for accurate hematological analysis of camelid blood.

Problem 1: The true volume of camelid RBCs is unknown

Despite all of the work that has been done to characterize the structure and molecular composition of camelid erythrocytes, the true volume of these cells is not known. This is an issue because all modern hematology analyzers, both electrical impedance and optical flow

cytometer based systems, determine cell counts in part by user-determined cell volume thresholds.[23] The first step in obtaining accurate automated RBC counts is therefore to know the expected cell volume of the species being analyzed. Automated flow cytometry analyzers are capable of directly measuring the volume of individual RBCs, [23-25] however this methodology is dependent on specific changes in cell shape that may not be observed in camelid RBCs as a consequence of their unique structural features (see below).

Manual methods for estimating mean RBC volume in a whole blood sample have been utilized to validate automated hematology methods in several species. [24,26,27] In most species, manual mean cell volume (MCV) calculations involve obtaining a RBC count from a validated method (i.e. counts from a hemacytometer or particle counter) and dividing this by the packed cell volume (PCV) of the blood sample obtained by simple centrifugation. Manual estimates of MCV in camelids have been reported, with the most recent studies providing reference intervals of 21-28 fL for alpacas and 21-31 fL for llamas, although these are still rather imprecise approximations.[3,28-30] Compared to other common domestic mammals such as the dog, the estimated camelid MCV range is lower. However, this finding is consistent with the results of visual side by side comparisons of camelid and human RBCs, in which the camelid cells appear consistently smaller in overall dimensions (length, width, and depth).[12]

Problem 2: Optical hematology analyzers use the Mie theory of light scatter to measure cell volume

The majority of automated hematology analyzers currently used in veterinary clinics and reference laboratories utilize flow cytometry to measure and differentiate blood cells. In flow cytometers, cells suspended in fluid pass single file through a narrow laser beam, and the cells

are identified and counted based on how they scatter light from the laser.[23] In the ADVIA 120 and 2120 hematology systems used in the present study, the two primary light scatter measurements for RBCs are low-angle (2-3^{o}) and high-angle (5-15^{o}) scatter, which translate into cell volume (in fL) and hemoglobin concentration (in g/dL), respectively.[25] The analyzer's interpretation of these low-angle and high-angle light scatter measurements is based on the mathematical principle of Mie theory, which works on the key assumption that the cell is a perfect sphere with homogeneous volume and refractive index.[24,25]

In the ADVIA 120/2120, before the RBCs interact with the laser beam, they are first exposed to the ADVIA RBC/PLT reagent, which is a proprietary combination of sodium dodecyl sulfate and glutaraldehyde fixative. This reagent interacts with the plasma membrane to convert the RBCs (and platelets) in the blood sample into isovolumetric spheres so that accurate direct volume and hemoglobin concentration measurements can be made for individual cells as they pass through the laser. These individual measurements are then averaged by the analyzer to provide the MCV and CHCM (corpuscular hemoglobin concentration mean) indices commonly used by clinicians when evaluating RBC disorders.[25] MCHC (mean corpuscular hemoglobin concentration) is another RBC index that is often used interchangeably with CHCM, although this value is calculated by the analyzer rather than derived from direct cell measurements.

Flow cytometry analyzers have been validated for several domestic animal species, and veterinary software is available with pre-set volume and hemoglobin concentration (HC) thresholds for a variety of species on the ADVIA.[31,32] However, there is evidence to suggest that camelid RBCs may not exhibit the deformability necessary to be transformed into isovolumetric

spheres by the ADVIA's RBC/PLT reagent. Khodadad, et al. performed a study in 1983 in which llama and human erythrocytes were exposed to a variety of conditions, including hyperosmolarity, ATP (adenosine triphosphate) depletion, and mixture with a non-ionic detergent. While human RBCs exhibited marked alterations in morphology in each of these scenarios, the llama RBCs remained essentially unchanged on SEM imaging, retaining their flat elliptical shape.[20] A separate study performed by Omorphos, et al. in 1989 also showed that camelid RBCs are resistant to shape changes induced by various drugs and metabolic conditions in RBCs of humans, and those of another artiodactyl outside of the Camelidae family (oryx).[5] In both studies, the resistance of the camelid erythrocytes to shape change was attributed to structural differences in their cell membranes, as described in the preceding section. To date, no research has been published to demonstrate a specific resistance to sphering by the RBC/PLT reagent, however based on these previous studies it seems highly unlikely that camelid RBCs would undergo this shape change.

Given that the analyzer's direct volume and HC measurements are dependent on light scatter properties of a homogeneous sphere, the ADVIA's MCV and MCHC indices are likely to be inaccurate for camelids, although it is unclear exactly how they would be affected. Various studies have been performed in an attempt to document the light scatter properties of ellipsoids[33] and non-spherical RBCs in different orientations,[34] and a "straight-ray approximation" has been proposed as an alternative method of volume measurement for non-spherical RBCs.[35] However, this information is still in the realm of basic physics, and is not currently practically applicable to the analysis of large numbers of RBCs in a patient sample.

The inaccuracies of MCV and MCHC values from automated analyzers have been documented in dehydrated human RBCs, and in human patients with decreased deformability of their RBCs.[36,37] In one of these studies, the use of an optical hematology analyzer on dehydrated RBCs resulted in an overestimation of MCHC and an underestimation of MCV. The authors acknowledged that inadequate sphering of the RBCs in these samples likely contributed to inaccurate measurements, and cautioned against interpreting RBC size and hemoglobin concentration based solely on automated measurements, recommending calculation of these values from a manual PCV instead.[37] Similar observations have been made in human patients with sickle cell anemia, in which irreversibly sickled RBCs exhibit decreased deformability (and likely incomplete sphering) as a consequence of polymerized abnormal hemoglobin bound to band 3 membrane proteins.[38] Hemograms from these individuals show a broader range of cell volume and hemoglobin concentration measurements, and it is suspected that automated MCV and MCHC measurements of these samples are also inaccurate.[37]

Problem 3: Species settings use cell volume to differentiate RBCs from platelets

Another challenge in camelid hemogram interpretation arises from a lack of certainty that the automated RBC count provided by flow cytometry analyzers represents the true RBC concentration in a given sample. The ADVIA 120/2120 systems use the same cytometer channel to analyze both RBCs and platelets (PLT). Cells with volumes between 0-200 fL and hemoglobin concentrations between 0-50 g/dL are documented on the RBC volume/HC cytogram and respective volume and HC histograms.[32] Since PLTs have volumes greater than 0 fL and lower refractive indices than RBCs, they are included in the bottom left corner of the RBC cytogram. The analyzer then divides the optical reading area for RBCs and PLTs at the 30 fL threshold – all cells with volumes greater than 30 fL are included in the RBC optical read area and counted as

RBCs, while cells with volumes between 0-30 fL are included in the PLT optical read area and PLT count, and mapped on a separate PLT cytogram.[32] The ADVIA uses "integrated RBC/platelet analysis" to differentiate large PLTs (volumes between 20-60 fL) from other small particles of similar size, such as microcytic RBCs, RBC fragments, and RBC ghosts. This analysis is performed on the basis of combined volume and refractive index measurements for individual cells.[25,32]

Accurate RBC and PLT counts are therefore primarily dependent on accurate cell volume measurements, which in turn are dependent on the cells undergoing adequate sphering reactions. While there is no evidence to suggest that camelid PLTs are not susceptible to sphering reactions in the RBC/PLT reagent, their RBCS appear to be resistant to this shape change, as described above. It therefore seems possible for a small RBC to be incorrectly sized and counted as a platelet, or vice versa. Additionally, since camelid RBCs are suspected to be smaller than those of other mammalian species, including humans, even an accurately sized RBC may fall into the PLT volume range if the appropriate species threshold is not used.[28,39] In human patients with microcytic anemia, the potential for overestimation of platelet counts by optical analyzers has been recognized.[40] One recent study identified the use of the reticulocyte channel as a method to improve the accuracy of optical platelet counts in these patients, since the platelets take up thiazole orange stain but the mature, microcytic RBCs do not.[40]

The ADVIA's veterinary software allows for the analysis of a blood sample according to multiple pre-defined species settings, which set different thresholds for the ideal volume and hemoglobin concentration measurements of RBCs. For example, the pre-determined volume threshold for the goat setting is lower than that for the dog setting, since goats are known to

have lower MCVs than dogs. Analyzing the same blood sample on multiple species settings will provide different absolute RBCs counts and RBC indices (MCV, MCHC, etc), as will manually altering the manufacturer's pre-set thresholds for a given species setting.[41] This suggests that if the true expected volume of camelid RBCs was known, the ADVIA could be used to provide accurate RBC counts. However, as stated in the first problem, the true volume of camelid RBCs is still uncertain.

Even though electrical impedance counters are less dependent on a specific cell shape than optical flow cytometers, they still use size thresholds to determine which particles to include in their counts, and to separate cell populations.[23,42,43] Overestimation of PLT counts by electrical impedance methods has been described in human patients with microcytic anemia, specifically those with MCVs less than 70 fL.[40] Knowledge of camelid RBC volume is therefore important for obtaining accurate RBC counts regardless of which automated instrument is utilized.

Problem 4: Automated cell volume measurements and RBC counts are used to determine other RBC values

Automated flow cytometry analyzers such as the ADVIA 120/2120 provide several RBC values which are the result of direct measurements of individual cells, including MCV and CHCM. Absolute RBC counts are also directly measured by the analyzer, as described above, although they are dependent on the initial cell volume measurement. Other values, such as hemoglobin concentration (HGB) of the blood sample, are the result of direct chemical analysis that is not dependent on cell size, shape, or number.

In the ADVIA 120/2120, HGB is measured spectrophotometrically after lysis of the RBCs.[25] The active ingredient in most RBC lysis buffers is ammonium chloride, which causes colloid osmotic lysis following diffusion of NH_3 into the cells, subsequent anion exchange via band 3 proteins, and ultimately net influx of chloride anions.[44] Studies utilizing various concentrations, volumes, and incubation times of ammonium chloride solutions have been performed with alpaca blood, however these studies do not describe an endpoint for measurement of complete hemolysis (or potentially a lack thereof).[45,46] A separate study using camel blood described incomplete lysis of the RBCs after incubation with an ammonium chloride buffer solution.[47] Additionally, protein electrophoresis of RBC membrane ghosts from humans and llamas shows residual hemoglobin in the llama membranes, suggesting incomplete lysis of the RBCs during ghost preparation.[20] However, additional research into the susceptibility of camelid RBCs to lysis with colloid osmotic buffers is lacking, and the specific composition of the ADVIA 120/2120 hemoglobin reagent is unknown. The hemoglobin concentrations measured by the ADVIA in the present studies appear physiologically appropriate, and are similar to those described by other investigators.[3,28] Ultimately, however, the degree to which camelid RBCs undergo hemolysis in the ADVIA 120/2120 hemoglobin reagent is uncertain, and warrants further investigation.

Additional values relating to erythron evaluation are calculated, rather than directly measured, by the analyzer. One of these calculated values is hematocrit (HCT), which is analogous to the sample's packed cell volume (PCV), a value that is often obtained manually after centrifugation of whole blood. The ADVIA calculates HCT as a product of the RBC count and the MCV. Therefore, if the measured RBC count and MCV of a sample are suspected to be inaccurate, then the analyzer's HCT calculation will also be inherently inaccurate. As mentioned briefly above,

most automated hematology analyzers including the ADVIA 120/2120 also provide a calculated mean RBC hemoglobin concentration (MCHC), which is often nearly identical to the measured CHCM in most species.[25] The MCHC is derived from division of the hemoglobin (HGB) concentration of the sample by the calculated HCT. While HGB may be unaffected by the other problems which plague automated camelid hematology analysis (see above), the MCHC value will be inaccurate in camelids due to its incorporation of the analyzer's calculated HCT. Previous studies have suggested measuring a manual PCV and substituting this value for HCT in the MCHC equation.[3,28]

Problem 5: Mean concentration of RBCs in healthy camelids exceeds the reportable range of the ADVIA

Automated camelid RBC counts from both flow cytometry and impedance analyzers are susceptible to error as a consequence of their dependence on accurate individual cell volume measurements, as described above. These values are therefore suspected to be inaccurate, although the degree of error and difference from the true value (bias) for each method are uncertain. Additionally, camelids are known to have a higher concentration of RBCs in their peripheral circulation (millions of cells/µL of blood) than many other species. Estimates of absolute RBC counts in camelids range from 10-17 million cells/µL, with some reports of counts approaching 18 million cells/µL.[3,4] This fact is problematic because the upper end of the reportable range for RBC counts on the ADVIA 120/2120 is 7 million cells/µL.[31]

Reportable range (also known as analytic measurement range), as defined by the Clinical Laboratory Improvement Amendment (CLIA) and the College of American Pathologists (CAP), is the "range of analyte values that a method can directly measure on the specimen without any dilution, concentration, or other pretreatment not part of the usual assay process."[48] This range

is determined by assessing an instrument's linearity, or the presence of a straight-line relationship between measured analyte value and the true specimen value (equivalent to a slope of 1 on an x vs. y scatterplot). The upper end of the reportable range is typically defined as the highest analyte concentration at which this linear relationship is observed, or in other words the highest instrument value that can be trusted as accurate for a particular analyte.[48] An important question when analyzing camelid blood is therefore whether the ADVIA will underestimate the RBC count when the true RBC concentration exceeds the instrument's reportable range. Additionally, because RBCs are present in such high concentrations in healthy camelids, it is possible that the cells would not flow single-file through the path of the laser beam, leading to a falsely decreased absolute count as multiple cells are counted as one. For this reason, it has been suggested that impedance particle counters might provide more accurate RBC counts than optical flow cytometry analyzers, although it is important that the correct size thresholds and sample dilutions are used, as described above.[3]

What is known currently

In summary, there is abundant evidence to suggest that automated complete blood count analysis in camelid species is inaccurate. However, validated methods to improve the accuracy of this analysis are lacking. Current recommendations include manual measurement of a PCV instead of using the ADVIA's HCT, and calculation of MCV and MCHC based on the PCV.[28] However, the MCV calculation includes the RBC count, so the accuracy of this calculated value is still dependent on obtaining an accurate absolute RBC concentration. To date, there is no validated, standardized method for obtaining a trusted RBC count in camelids, either by automated (flow cytometer or impedance) or manual (hemacytometer) methods, and previous studies reporting hematologic reference intervals for these species have utilized different

methods.[3,28,39] Several advanced techniques have been described in human hematology to aid in the characterization of RBC size and morphology in various disease states, however the practical applicability of these methods for analysis of large numbers of RBCs in clinical blood samples has yet to be determined.[49,50]

Mycoplasma haemolamae

Hemotropic mycoplasma (hemoplasma) infections have been described in numerous vertebrate species, with nearly global geographic distribution. Many of these wall-less bacterial organisms belong to the genus *Mycoplasma*, which combines organisms previously classified as *Hemobartonella* or *Eperythrozoon*.[51] Some hemotropic mycoplasmas, including *M. haemolamae*, have an additional qualifier of *Candidatus*, indicating that aspects of their biology are not fully described.[52] Throughout the remainder of this thesis, this organism will be referred to as *M. haemolamae*. *Mycoplasma* organisms have specific host ranges, but these organisms share similar morphologic characteristics in all host species, being observed epicellularly as small basophilic ring, coccoid, or rod-shaped structures on the surface of RBCs on Wright's stained blood smears, which occasionally cause slight indentations of the cell membranes. If there is a delay in blood smear preparation, the bacteria can fall off the RBCs ex vivo, leading to their appearance extracellularly in the background of the smear, and potentially increasing the likelihood that they may be mistaken for artifact.[52] In recent years, sequencing of the *M. haemolamae* genome has allowed for development of a polymerase chain reaction (PCR) assay for more definitive diagnosis of infection in camelid blood samples.[53,54]

Clinical disease caused by the hemotropic mycoplasmas often involves anemia. Clinical anemia due to extravascular hemolysis is observed with hemoplasma infections in cats and pigs.[52,55] In other species (dogs, ruminants, camelids), splenectomy or immunologic compromise is often necessary to observe clinical disease in bacteremic animals.[51,52] Hypoglycemia has also been noted in cases of clinical *Mycoplasma* infections, although the pathophysiology of this finding is uncertain.[52] In camelids, clinical anemia (defined as decreased PCV in most reports) has been described in cases of *M. haemolamae* infections, although clinically affected animals are usually young, stressed, or debilitated by a concurrent illness (such as gastrointestinal parasitism or malnutrition), or otherwise immunosuppressed. Clinical disease in otherwise healthy, adult animals infected with *M. haemolamae* appears to be uncommon.[3,52,56,57]

In most species, transmission of these organisms is presumed to occur via arthropod vectors (fleas, ticks, etc), however supporting evidence for this presumption is lacking.[52] Vertical transmission of *M. haemolamae* is also suggested in camelids based on reports of bacteremia with or without clinical disease in 1- and 4-day old crias.[58,59] In the 1-day old llama, neither the cria nor the dam were anemic, however large numbers of Mycoplasma organisms were observed on the cria's blood smear. Organisms were not observed on a blood smear from the mother, although PCR assays were not available at the time of this report to definitively exclude the possibility of maternal bacteremia.[58] In contrast, the 4-day old alpaca was anemic with large numbers of organisms observed on a blood smear. The dam in this case was not anemic and did not have organisms on a blood smear, but both the dam and cria had PCR evidence of *M. haemolamae* infection.[59]

The development and increasing availability of the *M. haemolamae* PCR assay has allowed for assessment of the prevalence of this infection in camelid populations, which appears to vary substantially based on geographic location and likely other husbandry-related factors. In a 2010 study of alpacas and llamas in Chile and Peru, the prevalence of infection ranged from 9-19%.[56] A slightly higher prevalence was identified in an alpaca population in England (29%),[60] while a much lower prevalence was described in a group of 206 alpacas in New Zealand (0.97%).[61]

Despite their utility in diagnosing the presence of the bacteria, interpretation of *M. haemolamae* PCR results can be difficult. As noted above, clinical illness secondary to this infection is uncommon in otherwise healthy adult camelids, so the significance of a positive PCR assay is uncertain. If the animal is healthy, is it at increased risk of developing anemia? If the animal is anemic, is the anemia a direct consequence of *M. haemolamae* infection, or is there evidence of a concurrent disease process (i.e. gastrointestinal parasitism or systemic inflammation) that may be contributing to the decreased RBC mass? Additionally, there is experimental evidence which indicates that infected alpacas may remain PCR positive for *M. haemolamae* after treatment with oxytetracycline, despite the clearance of visible organisms from blood smears and resolution of clinical anemia.[62] Additional research is needed to further investigate the relationship between *M. haemolamae* PCR status and RBC values in cases of apparent subclinical infection.

Camelid parasitology

Gastrointestinal (GI) nematode infestations represent another common source of potential clinical illness in camelid species living in the southeastern United States. Commonly identified nematodes include: *Haemonchus contortus*, *Trichostrongylus axei*, *Teladorsagia circumcincta*,

Ostertagia ostertagi, *Lamanema chavezi*, *Nematodirus batti*, *Aonchotheca* (*Capillaria)* spp., *Trichuris* spp., and *Oesophagostomum* spp.[63] Of these parasites, only *Lamanema chavezi* is specific to llamas and alpacas; the remainder are also found in small and/or large ruminant species.[63,64]

The clinical significance of many of these nematodes in camelids is uncertain, with the exception of *H. contortus*, which is a strongylid nematode known to cause severe hemorrhagic anemia. The pathogenesis of *H. contortus* infections has been well-defined in sheep,[65] and recently in llamas and alpacas.[66] *H. contortus* resides in the abomasum of sheep and goats, and in the third gastric compartment (C3) of camelids. Whole blood is lost as the worms feed on the mucosal blood supply, as well as secondary to mucosal irritation. Clinical signs of anemia often include mucous membrane pallor, weight loss, and submandibular edema.[66]

In contrast to *M. haemolamae* infections, in which the relationship between PCR status and susceptibility to clinical disease is uncertain, the link between *H. contortus* infections and clinical anemia is well known. Several methods exist for diagnosis and differentiation of GI nematode infections, including identification of adult worms within the GI tract at necropsy, various fecal flotation and egg counting methods, fecal culture, PCR, and lectin staining.[63,66,67] Fecal flotation and calculation of a fecal egg count (FEC) is one of the most common ante-mortem diagnostic methods for GI nematodes, and is also commonly used as a screening test at the herd level to determine the proportion of animals likely to be shedding large numbers of eggs onto the pasture. Screening of animals within a herd based on body condition scoring and FAMACHA scores (see below) is currently recommended in order to allow for targeted deworming of

animals likely to have high egg counts, since unnecessary deworming of the entire herd is likely to decrease refugia and promote resistance of the parasites to commonly used medications, limiting the efficacy of these treatments when animals become clinically ill.[68]

The modified McMaster technique for obtaining FECs is most often described, in which a fecal sample is mixed with a flotation solution and then loaded into a counting chamber. Microscopic evaluation of the chamber allows for the visual identification of egg types (strongylid vs. other nematode vs. coccidia, etc). Variations of this technique have been reported, although the use of a sucrose flotation solution with a specific gravity of 1.200-1.350, and a chamber volume of 1.0 mL seem to yield the highest stronglyid egg counts in both sheep and camelids.[67,69] In addition to identifying the presence of a parasite infestation, an FEC also provides a semi-quantitative assessment of the animal's nematode burden, since a larger number of adult female worms in the gastric compartment would be expected to produce a proportionally larger number of eggs.[65,70] Although strongylid eggs of all species appear similar on flotation, necessitating the use of additional techniques (larval culture, lectin staining, etc) to differentiate them, *H. contortus* is the nematode which is most commonly present, and of most clinical concern.[66] Additionally, both FEC and adult worm numbers have been shown to exhibit a linear relationship with the amount of blood loss in sheep experimentally infected with *H. contortus*.[70]

The FAMACHA system (named for its inventor, Dr. Francois "Faffa" Malan) is another method commonly associated with diagnosis of GI nematode infections in small ruminants and camelids. The conjunctival mucous membrane color of an animal is compared to a standardized card with five shades of pink (ranging from white to bright pink-red). This is used to provide an estimate

of the severity of the animal's anemia and its relative need for deworming medication, given that *H. contortus* infection is the most likely cause of clinical anemia. Pale white mucous membranes correspond to a score of 5, which is the most severe degree of anemia. Bright pink mucous membrane color would receive a score of 1 (clinically normal). In one study in sheep and goats, a FAMACHA score of 3 correlated to an FEC of approximately 1000 eggs per gram (epg), although there appears to be substantial geographic variation in typical FEC ranges.[71] This system has high specificity and fair sensitivity for detection of anemia in sheep and goats, [71] and in alpacas and llamas, [72] and there is good correlation between FAMACHA scores and HGB and HCT measurements of peripheral blood.[73]

Positive correlation has also been demonstrated between FEC and PCV in clinically ill alpacas and llamas, [66] and in experimental and natural *H. contortus* infections in sheep.[73,74] To date, no research has been done to determine whether there is a correlation between FEC and RBC values in clinically healthy camelids, and there is also a lack of certainty in the interpretation of a clinically significant FEC value in otherwise healthy animals. In one study performed in the southeastern United States, the majority of anemic camelids had an FEC of ≥1000 epg, [72] while in another study performed in New Zealand a fecal egg count of 250 epg was reported as "high and clinically significant."[61]

CHAPTER I
AUTOMATED VS MANUAL HEMATOLOGY METHODS

Abstract

Background: Accurate measurement of erythrocyte values by automated hematology analyzers requires isovolumetric sphering of the cells. Camelid erythrocytes have unique membrane characteristics, which make them resistant to sphering. Validation of an optimized method for hematologic analysis of camelid blood has not been published.

Objectives: Demonstrate whether camelid erythrocytes sphere in the ADVIA RBC/PLT reagent (a sphering reagent), and determine the optimal ADVIA setting for camelid CBC analysis.

Methods: Camelid and canine blood were each added to ADVIA RBC/PLT reagent or saline at a 1:625 dilution, and evaluated by light microscopy for erythrocyte sphering. Camelid blood (n = 25) was analyzed on a Z2 Coulter counter using one or more lower volume thresholds (4, 5, 6, 8, 10, 12 fL) to determine the optimal threshold for manual RBC quantification. Additional camelid blood (n = 132) was analyzed on an ADVIA 120 hematology analyzer using one of three settings (bovine, equine, camelid). The analyzer's playback feature was used to obtain data using the other two settings. The playback feature was also used to obtain data using the goat setting on a subset of samples. A manual packed cell volume (PCV) was measured, and manual calculations performed for mean cell volume (MCV) and mean cell hemoglobin concentration (MCHC). RBC values (RBC count, MCV, MCHC, and HCT) were compared between all ADVIA settings, and between each setting and manual calculations using Passing-Bablok regression and Bland-Altman plots.

Results: Canine erythrocytes sphere when mixed with ADVIA RBC/PLT reagent, while camelid erythrocytes do not. The ADVIA 120 equine and camelid settings are identical and provide the closest approximations to manual estimates of erythrocyte and platelet counts. MCV, MCHC, and HCT from all settings are significantly different from manual calculations. The 8 fL threshold

of the Z2 Coulter counter yields the highest RBC count, although some platelets may be included in the count.

Conclusions: Camelid erythrocytes do not sphere, resulting in inaccurate erythrocyte values from automated hematology analyzers. Optimal determination of CBC data requires a combination of automated and manual methodologies.

Background

Results from an automated veterinary hematology analyzer are highly dependent on the species setting that is used to analyze the blood sample, as these settings must be optimized to account for differences between species. This is especially true for values related to erythrocytes and platelets. It has historically been difficult to obtain accurate hematology results for camelid species, since their red blood cells (RBC) and platelets are characterized by unique features that are not present in more commonly studied domestic animals, including elliptically-shaped RBCs and extremely small platelets. These characteristics can affect the way that the cells are identified and measured by automated analyzers, making optimization of a camelid setting on any hematology analyzer a challenge. To date, there are no published validation studies identifying optimal settings for camelids, although anecdotal reports indicate that the equine setting of the ADVIA 2120 is preferred.[28,39]

Camelid erythrocyte structure and function

Camelid red blood cells are small, flat, and elliptical, with a rigid cell membrane. It is thought that these characteristics aid in adaptation to living at high elevations and in arid environments, by providing increased oxygen delivery and resistance to osmotic lysis, respectively.[2] Specific differences in the camelid erythrocyte plasma membrane, including relative increases in the

proportions of certain proteins, result in decreased erythrocyte deformability and a consequent lack of shape change when exposed to a variety of substances that alter membrane lipids in vitro in other species.[5] While this resistance to deformation likely increases the physiologic resistance to erythrocyte lysis during periods of dehydration, it also presents challenges to our ability to accurately identify and quantify camelid RBCs using conventional automated hematology methods.

Hematology analyzer methodology

Automated optical flow cytometry analyzers such as the ADVIA 120/2120 (Siemens Healthcare Diagnostics, Tarrytown, NY) identify and quantify RBCs and platelets based on species-specific volume and hemoglobin concentration thresholds. However, the accurate determination of these parameters requires the cells to be isovolumetrically sphered (i.e. turned into spheres without changing their cell volume). This is accomplished in samples from species with biconcave disc-shaped RBCs by the addition of a solution of sodium dodecyl sulfate (SDS) and subsequent fixation with glutaraldehyde.[25] SDS and glutaraldehyde are the main components of the ADVIA RBC/PLT reagent.[32] Since cell shape is eliminated as a variable in spherical cells, their light scatter properties can be assessed based on the Mie theory of light scatter by spheres, and converted to measurements of volume as well as internal complexity (refractive index – translated by the analyzer into hemoglobin concentration).[25] Since cell volume measurements from optical analyzers are used to identify a cell as an RBC (vs. a platelet) and to determine the absolute RBC count and mean cell volume (MCV), the accuracy of these values is highly dependent on this sphering reaction. Additionally, other RBC values including mean cell hemoglobin concentration (MCHC) and hematocrit (HCT) are based on RBC and MCV measurements, and are therefore affected by any inaccuracies in these measurements. Camelid

RBCs are also smaller than those of most other domestic species. The estimated MCV of alpaca RBCs was 21-28 fL in one study,[28] while the typical canine MCV is often greater than 60 fL. As a result, there is increased risk of RBCs being counted as platelets by the ADVIA if their volume measurement is inaccurate or the incorrect species thresholds are used. Based on knowledge of camelid erythrocyte morphology and membrane structure, as well as on prior studies,[5] it is reasonable to hypothesize that camelid RBCs will be resistant to sphering with the ADVIA's RBC/PLT reagent, and that RBC quantification and associated indices obtained from the ADVIA 120 will differ significantly from their true values as determined by other manual and automated methods.

As there is currently no validated camelid setting for the ADVIA 120/2120, nor a way to predict the degree or direction of any inaccuracies introduced by a lack of RBC sphering, the goal of this study was to determine which ADVIA species setting (bovine, equine, or goat) would provide the most accurate CBC values related to RBCs and platelets, as well as to determine the bias between ADVIA RBC indices and those obtained with manual methods. It was hypothesized that absolute RBC and PLT counts will vary significantly between different species channels on the ADVIA, and that the optimal method for determining accurate CBC data will require a combination of automated and manual methods. Results of this study are intended to aid in the development of more accurate camelid hematology reference intervals, and potentially in the design of future studies of certain related hematologic disorders, such as canine hereditary elliptocytosis.

Materials and Methods

RBC sphering

Given that the ADVIA's measurement of MCV and quantification of RBCs is dependent on proper RBC sphering, if camelid erythrocytes are resistant to sphering then CBC results from the ADVIA will be inaccurate regardless of which species setting is used. One EDTA (ethylenediaminetetraacetic acid)-anticoagulated sample each of canine and camelid whole blood were used to prepare serial dilutions in both the ADVIA RBC/PLT reagent and normal saline (as a control). The specific composition of the RBC/PLT reagent is proprietary information; however, its key ingredients are sodium dodecyl sulfate and a glutaraldehyde fixative.[25] Canine and camelid blood samples from obtained from hospital patients who were not anemic based on laboratory reference intervals, where blood had been collected as part of their clinical evaluation. A final dilution of 1:625 (blood to reagent or blood to saline) was used [David Zelmanovic, personal communication]. The total volumes of the dilutions were multiplied by a factor of 3 via trial and error to increase the final volume of the dilution while maintaining the desired final concentration of 1:625, since the sphering of normal human RBCs is dependent on the presence of a volume of reagent sufficient to completely coat the surface of each cell.[75] For example:

1st dilution: 10 µL blood to 240 µL reagent/saline = 1:25

2nd dilution: 10 µL 1st dilution to 240 µL reagent/saline = 1:625

Multiply volume by a factor of 3:

1st dilution: 30 µL blood to 720 µL reagent/saline = 1:25

2nd dilution: 30 µL 1st dilution to 720 µL reagent/saline = 1:625

A drop of each final dilution was placed on a slide with a coverslip, and immediately examined with light microscopy at 500x magnification to evaluate for RBC sphering.

RBC linearity of the ADVIA

The range of absolute RBC counts measured by the ADVIA is 0-7,000,000 cells/µL, according to the ADVIA 120 performance specifications.[31] However, camelid blood has an erythrocyte concentration that is 2-3 times that of humans and other domestic mammals, and healthy llamas are reported to have RBC counts up to 18,000,000/µL, far exceeding the technical specifications of the ADVIA.[3,29] In order to evaluate the ADVIA's ability to provide accurate RBC counts above the manufacturer's specified range, the linearity of the ADVIA 120 was assessed through serial dilution of equine blood. Equine blood was used because the equine setting of the ADVIA has been validated for horses, and because horses have relatively low MCVs among the common domestic species (<60 fL), allowing for artificial concentration of a blood sample to an absolute RBC count within the anticipated reference interval of camelid species without exceeding a supraphysiologic PCV.[4,27]

An EDTA-anticoagulated equine whole blood sample with an ADVIA 120 RBC count of 6,220,000/µL was concentrated via centrifugation at 3500 rpm for 3 minutes. The packed RBCs in the centrifuged sample were re-suspended in a small volume of autologous plasma, and analyzed in duplicate using the ADVIA 120. This concentrated sample was then serially diluted into four additional pools using increasing volumes of autologous plasma – 4:1, 3:2, 2:3, and 1:4 (packed RBCs to plasma). These four pools were measured in triplicate on the ADVIA 120, as was a sample of autologous plasma. Measured RBC counts for each dilution level were

averaged, and linear regression analysis was used to assess the correlation of the measured and expected values for each dilution.

Determination of appropriate RBC size thresholds – part I

In order to determine which cell volume threshold would be most likely to provide accurate RBC and platelet counts, camelid whole blood was analyzed using a Z2 Coulter particle counter (Beckman Coulter Inc., Fullerton, CA). Compared to the ADVIA, which identifies and counts cells based on their light scatter properties (volume and refractive index), the Z2 counts cells as particles that cause a measurable change in electric resistance as they flow across an aperture within the analyzer.[42] Unlike the ADVIA and similar optical flow cytometry methodologies, which require RBCs to be isovolumetrically sphered in order to be correctly sized and counted, cell shape does not affect Z2 particle counts. Instead, lower and upper volume thresholds are set by the operator, and all cells (particles) whose volumes fall between these thresholds are counted. This "aperture-impedance principle" of cell counting has been identified as a reference method for red and white blood cell counting and sizing in human hematology,[43] as it is both more precise and accurate than manual methods such as hemacytometer chamber counting.

Venous whole blood was collected from a group of camelids (adult llamas and alpacas) in New York State between November 2009 and October 2015. This group included both clinically healthy animals, as well as those presenting for various disease conditions. Blood was collected into K_2EDTA tubes, and transported to the clinical pathology laboratory at Cornell University College of Veterinary Medicine. Blood from healthy animals was collected with informed owner

consent according to approved IACUC protocols. Blood from ill animals was collected as part of their clinical evaluation.

Samples were analyzed on the Z2 counter at various lower volume thresholds: 4 fL, 5 fL, 6 fL, 8 fL, 10 fL, and 12 fL. According to the International Council for Standardization in Hematology, the ideal lower threshold for RBC counts should be set between the volumes of platelets and red blood cells,[43] so as to exclude as many platelets and include as many RBCs as possible in the count. Since the exact volume of camelid RBCs is unknown and the degree of overlap with platelet volume (if any) is uncertain, all samples were run using at least two of the size thresholds listed above, in an attempt to identify the ideal RBC size threshold. All samples were analyzed at the 8, 10, and 12 fL thresholds, and after preliminary analysis the data for the lower thresholds (4, 5, and 6 fL) were excluded based on the apparent inclusion of two distinct cell populations (RBCs and platelets) on the Z2 histograms.

Briefly, blood samples were prepared for analysis by creating two serial dilutions in an isotonic electrolyte solution (Z Pak, Beckman Coulter Inc., Fullerton, CA). The first dilution was prepared by pipetting 20 mL of solution into a cuvette (Accuvette, Beckman Coulter Inc., Fullerton, CA), removing 40 µL of the solution and then adding 40 µL of well-mixed whole blood (1:500 dilution). 20 mL of electrolyte solution was added to a second cuvette, from which 200 µL was removed and replaced with 200 µL of sample from the 1:500 dilution (final dilution: 1:50,000). The 1:50,000 dilutions were used to perform RBC counts in duplicate at each lower volume threshold. An upper volume threshold of 120 fL, an aperture diameter of 100 µm, and a resolution of 256 nm were held constant for all Z2 counter analyses.

A CBC analysis was also performed on each sample using the ADVIA 2120 equine setting (20-70 fL RBC volume threshold), and the ADVIA's playback feature was used to obtain results on the bovine (5-65 fL) and goat (0-50 fL) settings.

A manual PCV was also measured on each sample, using a microhematocrit centrifuge for 10 minutes at 13,200 g.

Determination of appropriate RBC size thresholds – part II

As described above, the cell counts obtained from the Z2 Coulter counter are a reflection of the number of particles in a sample within a specified volume range, without providing any additional information regarding the identity of the particles. In species with known RBC volume, one can be reasonably confident that a whole blood sample analyzed with the appropriate size thresholds will yield an accurate RBC count. However, given that normal RBC volume is unknown in camelid species, it is also unknown how closely Z2 RBC counts reflect the true RBC count in a given animal. For example, if the lower volume threshold is too high, a proportion of smaller RBCs may be excluded from the total count. Additionally, if the lower threshold is too low, platelets may be included, falsely increasing the RBC count. In order to further validate the Z2 Coulter counter as a reference method for camelid RBC counts and size determination, additional blood samples were obtained from four clinically healthy, non-anemic llamas (PCV ≥ 22%, the lower limit of the camelid reference interval at Cornell CVM) and separated into RBCs and platelet-rich plasma (PRP). Whole blood, RBCs and PRP from each animal were analyzed on both the Z2 counter (at various lower thresholds) and the ADVIA 2120 (various species settings). The Z2 lower volume threshold yielding the highest count on the RBC sample and the lowest count on the PRP sample would be expected to represent the most accurate volume threshold for camelid RBCs.

Whole blood

Each whole blood sample (9 cc) was collected into a 20 cc plastic syringe containing 1 cc of acid citrate dextrose (ACD) as an anticoagulant, as this is the recommended anticoagulant for use in preparation of platelet-rich plasma.[76] The sample was well-mixed by gentle inversion, and transferred into a 15 cc polypropylene conical tube. CBC analysis was performed on each whole blood sample using the ADVIA 2120. Each of the four samples was initially run on one of three species settings: bovine, goat, and equine, and the instrument's playback feature was used to obtain results for the other two settings. The initial setting was determined randomly for each sample. A blood smear was prepared from each sample, and a manual PCV was measured, as described above. Based on preliminary results from part I of this portion of the study, each whole blood sample was also analyzed on the Z2 Coulter counter at three lower volume thresholds: 8 fL, 10 fL, and 12 fL. The upper volume threshold, resolution, and dilutions were held constant across all lower threshold settings, as described above. Each sample was measured 2-4 times each, depending on available sample volume, and the mean of the measurements was calculated.

Platelet-rich plasma

Platelet-rich plasma (PRP) was prepared according to a previously described protocol.[76] The conical tube containing the remaining whole blood was centrifuged at 720 x g for 15 minutes at 21^{o}C. The plasma was removed, leaving the buffy coat and RBCs at the bottom of the tube. The plasma (PRP) was analyzed on the ADVIA 2120 using the same species setting as for the patient's whole blood sample, and a modified Wright's stained direct smear was prepared to verify the presence of platelets and relative absence of RBCs and WBCs. The PRP was also

analyzed on the Z2 counter as described above, at each of the three lower volume thresholds (8, 10, and 12 fL).

The remaining PRP sample from each animal was centrifuged a second time at 720 x g for 15 minutes. The supernatant was removed and centrifuged in an Eppendorf tube at 13,000 rpm for 5 minutes. The supernatant from this hard spin (platelet-poor plasma, PPP) was analyzed on the ADVIA 2120 and at each of the lower volume thresholds on the Z2 counter.

Platelet-poor RBC suspension

The buffy coat and a small portion of the top of the RBC layer was removed from the remaining RBCs in the conical tube, and discarded. In an Eppendorf tube, 650 µL of PPP and 260 µL of concentrated RBCs were combined and gently mixed, in order to obtain a platelet-poor RBC sample with a similar PCV to that of the original whole blood sample. A manual PCV was performed. A blood smear was prepared from this reconstituted RBC sample, and stained with modified Wright's stain to microscopically verify the presence of RBCs and relative absence of platelets and WBCs. The sample was also analyzed once on the ADVIA 2120 using the sample's original species setting, and on the Z2 counter at the 8, 10, and 12 fL lower volume thresholds. Additionally, a portion of the reconstituted RBC sample was mixed with an equal volume of both PRP and PPP (i.e. 200 µL of RBCs plus 200 µL of PRP or PPP). Each of these samples was also analyzed on the ADVIA 2120 and the Z2 counter, and a manual PCV was performed as described above. The sample types and analyses performed on each sample are summarized in Figure 1. All tables and figures are located in the Appendix.

Patient selection and sample collection

Venous whole blood was collected from clinically healthy adult (≥ 1-year-old) alpacas and llamas from 12 farms in eastern Tennessee between July and October 2015. Health was defined as a lack of significant abnormalities on physical examination as determined by an experienced veterinarian, a FAMACHA score of 3/5 or lower,[72] a body condition score (BCS) of 2.5/5 or higher, and no history of illness or injury within the previous 3 months. All examinations and sample collections took place on the farms, and informed client consent was obtained for all procedures (approved UT IACUC protocol 2298-0914).

A maximum of 12 animals were collected per farm. Individual and herd histories were obtained, including species, age, sex, pregnancy/breeding status, housing, diet, vaccines, and parasite control practices. After examination of each animal, a maximum of 10 mL of blood was collected by an experienced veterinarian from an external jugular vein using a 12 mL plastic syringe (Covidien LLC, Mansfield, MA) attached to a 20 gauge, 1.5-inch needle (Covidien LLC, Mansfield, MA). The blood was placed in a 4 mL K_2EDTA tube (Becton Dickinson, Franklin Lakes, NJ) and transported to the University of Tennessee clinical pathology laboratory in a cooler that was maintained at 4^{o}C until arrival. Samples were promptly analyzed by trained laboratory personnel, with all analyses completed within 6 hours of sample collection. Samples were evaluated for appropriate filling of the EDTA tube, and for clot formation and/or gross lipemia. Underfilled, clotted, and lipemic samples were excluded. A CBC with reticulocyte count was performed on each sample using the ADVIA 120 hematology instrument according to laboratory standard operating procedures (SOP). One of three species settings was used:

Bovine: RBC volume threshold of 5-65 fL

Equine: RBC volume threshold of 20-70 fL

Modified equine (referred to hereafter as Camelid): RBC volume threshold of 5-70 fL

The true MCV of camelid RBCs in health is not known, although previous studies suggest a range of 21-28 fL based on manual calculations.[28] Although the camelid setting is not a manufacturer-defined species setting on the ADVIA, it was created in the UT clinical pathology laboratory because the lower end of the size threshold in the equine setting is very close to the expected size of camelid RBCs. The lower size threshold of the camelid setting is hypothesized to allow better enumeration of smaller camelid RBCs. This may become important if analyzing samples from camelids with conditions known to cause microcytosis, e.g. iron deficiency, since these small RBCs might be excluded from analysis on the equine setting. Although the size thresholds for the camelid and bovine settings overlap substantially, pilot data for this study revealed significant differences in the RBC values between these two settings (data not shown). This suggests that RBC evaluation by the ADVIA is dependent on factors other than RBC volume alone (i.e. hemoglobin concentration thresholds).

The patient samples were randomly and equally distributed among the three settings to eliminate potential bias, and the playback feature of the ADVIA was used to adjust each animal's results for the other two settings. For example, if a sample was run on the equine setting, playback was used to obtain CBC results for the bovine and camelid settings, so that each physical sample was only measured once. After all samples had been collected and analyzed, a subset of the samples was also randomly selected (using a random number generator) to be analyzed on the ADVIA's goat setting (RBC volume threshold of 0-50 fL) using the playback

feature. Manual PCVs were determined following one minute of centrifugation in a rapid fixed angle head microhematocrit centrifuge (HemataStat II, EKF Diagnostics, Boerne, TX). The centrifuge's "read" function was used to determine the PCV; this function is calibrated for the microhematocrit's centrifuge speed to provide a PCV that is comparable to a traditional microhematocrit centrifuge, but with results obtained more rapidly. Proper microhematocrit centrifuge function was verified using an electronic tachometer prior to beginning the study, and a series of test centrifugations of camelid blood (data not shown) confirmed there is no significant difference in PCV with centrifugation times longer than one minute. ADVIA 120 and manual PCV analyses were performed by a licensed medical technologist trained in instrument use and following laboratory SOPs. The ADVIA 120 underwent daily quality control using 3-level QCM (OPTIpoint, Siemens Healthcare Diagnostics, Tarrytown, NY).

A minimum of one blood smear was prepared from each sample, and reviewed by a medical technologist according to laboratory SOP. Blood smear review included a leukocyte differential, RBC morphology review, and a manual platelet estimate. Blood smears were also reviewed by a board-certified veterinary clinical pathologist.

Additionally, venous whole blood samples were collected from camelid patients (alpacas and llamas) presenting to the University of Tennessee Veterinary Medical Center's large animal hospital with various clinical complaints between January and May 2016, if a CBC was part of the attending clinician's diagnostic plan. For these animals, blood samples were collected, transported to, and processed by laboratory personnel according to the large animal clinic and clinical pathology laboratory SOPs. The CBC (and reticulocyte count, if requested by the

clinician) was run using the ADVIA 120 camelid setting, and a manual PCV and blood smear review were performed. CBC results were also obtained on the bovine and goat settings using the ADVIA's playback feature.

Statistical analyses

Statistical analysis was performed using commercial software (MedCalc Statistical Software version 17.4 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2017). The distribution of RBC and PLT variables from all analyses was assessed for normality using the D'Agostino-Pearson test. Repeated measures ANOVA with pairwise comparisons were performed to compare the RBC counts obtained from the three Z2 lower volume thresholds and from each of the ADVIA 2120 species settings (equine, bovine, and goat). Repeated measures ANOVA with pairwise comparisons were also performed between the 8, 10, and 12 fL threshold Z2 particle counts for platelet-poor RBC, PRP, and whole blood RBC counts to determine whether the counts obtained at each threshold differed significantly from one another. The lower Z2 threshold yielding the highest RBC counts while appearing to include the fewest platelets was used as the reference method for all future comparisons to the ADVIA and manual methods.

A manual mean cell hemoglobin concentration (MCHC) was calculated using the ADVIA's hemoglobin (HGB) measurement and the manual PCV for each EDTA whole blood sample. Calculated MCV values were also determined, using the manual PCV and RBC counts obtained from the Z2 counter. Mean and median differences between the ADVIA and manual methods (Z2 RBC count, calculated MCHC, and calculated MCV) were determined, as well as the range of differences, and the proportion of ADVIA values within the allowable total error (TE_a) range of

the manual methods. The TE_a ranges for the RBC values were based on the American Society for Veterinary Clinical Pathology's (ASVCP) guidelines for hematology measurands.[77] Passing-Bablok regression and Bland-Altman difference analysis were performed to assess for the presence of bias between the ADVIA's hematocrit value (HCT) and manual PCV, the ADVIA's RBC count for each species setting and the reference Z2 count, the ADVIA's MCHC for each species setting and the calculated MCHC, and the ADVIA's MCV for each species setting and the calculated MCV. Additionally, bias was calculated between the MCV calculated from the ADVIA's equine RBC count and the MCV calculated from the reference Z2 count. The ADVIA's platelet count for each species setting was compared to manual platelet estimates from blood smear review using the Wilcoxon test for paired samples.

Results

RBC sphering

Canine RBCs exhibit sphere formation when incubated with ADVIA RBC/PLT reagent, and retain a biconcave disc shape when incubated with normal saline, as examined by light microscopy. In contrast, alpaca RBCs maintain a flat ellipsoid shape in both RBC/PLT reagent and normal saline (Figure 2), and do not exhibit sphere formation.

RBC linearity of the ADVIA

The mean measured RBC count of the equine autologous plasma sample was 330,000/µL (minimum), and the mean measured RBC count of the concentrated packed RBC sample was 14,115,000/µL (maximum). The maximum measured RBC count was within the laboratory reference interval for camelids at the University of Tennessee (7,900,000-17,000,000/µL). The serial plasma to packed RBC dilutions yielded measured RBC counts between these minimum and maximum values (Table 1). Measured RBC counts were compared to expected RBC counts

(based on the sample dilution), and the coefficient of determination (R^2) for the correlation of measured RBC counts vs. expected RBC counts was 0.998 ($p<0.0001$) (Figure 3).

Determination of appropriate RBC size thresholds

EDTA-anticoagulated whole blood was collected from 25 camelids, four of which were anemic based on a laboratory PCV reference interval of 22-45% (Cornell University). All samples were analyzed on the 8, 10, and 12 fL thresholds of the Coulter Z2 counter and on the equine, bovine, and goat channels of the ADVIA 2120. The highest mean RBC counts were obtained with the 8 fL lower threshold of the Z2 counter, compared to the 10 and 12 fL thresholds. The mean RBC counts were significantly different ($p<0.0001$) between the three lower Z2 thresholds, however there was overlap between the 95% confidence intervals for all means (Figure 4). The highest mean RBC count on the ADVIA was obtained on the equine channel, and the lowest mean RBC count was obtained on the bovine channel. All ADVIA RBC counts were significantly different from all lower Z2 threshold RBC counts, with the exception of the ADVIA equine RBC count and the 12 fL Z2 count ($p=0.785$). However, there was overlap between the 95% confidence intervals for all mean RBC counts (ADVIA and Z2) (Figure 4).

ACD-anticoagulated whole blood was collected from four llamas, and analyzed as summarized in Figure 1. The ADVIA, Z2, and PCV results for the original whole blood, PRP, PPP, and platelet-poor RBC samples are listed in Table 2. When the four platelet-poor RBC samples were analyzed on the 8, 10, and 12 fL lower thresholds of the Z2 counter, the highest mean RBC count was obtained with the 8 fL threshold, however there were no significant differences between the three lower Z2 thresholds ($p>0.05$) (Figure 5).

When the PRP samples were analyzed on the 8, 10, and 12 fL lower thresholds of the Z2 counter, the lowest mean platelet count was obtained on the 12 fL threshold, and the highest mean platelet count on the 8 fL threshold. Particle counts were significantly different between the lower Z2 thresholds ($p = 0.015$), however all PRP Z2 counts were significantly lower than the ADVIA platelet counts (all species settings) ($p<0.0001$) (Figure 6).

The 8 fL lower threshold of the Z2 counter yielded the highest RBC particle counts on the platelet-poor RBC suspensions, however it is suspected that platelets may be included in these counts based on the results from the PRP experiments. The 10 fL lower Z2 threshold yielded slightly lower counts for the platelet-poor RBC suspensions, but also provided slightly lower counts for the PRP samples, increasing confidence in the RBC count estimates. The 12 fL Z2 threshold provided the lowest RBC counts and the highest platelet counts, and was suspected to underestimate the true RBC count. Since there was no significant difference between the Z2 RBC counts at the three lower thresholds, the 10 fL lower threshold was selected as the reference method for RBC count, as it was suspected to include fewer platelets than the 8 fL lower threshold. The reference method for MCV was a calculated value utilizing this RBC count. RBC counts and MCV values obtained from the different ADVIA species settings (equine, bovine, and goat) using the 25 EDTA-anticoagulated camelid whole blood samples were compared to the results from these reference methods in order to identify the most accurate ADVIA setting. Comparisons included the mean and median differences between the methods, the range of differences, and the proportion of ADVIA results falling within the allowable total error (TE_a) range for the reference method. Passing-Bablok regression analysis and Bland-Altman difference plots were also used to assess the bias of the ADVIA RBC and MCV measurements

compared to the reference methods. No differences were observed between the ADVIA equine and camelid settings for any RBC or platelet values, and the equine setting results were used for all comparisons.

Overall, the RBC counts on the ADVIA were lower than the 10 fL Z2 particle counts for whole blood, regardless of the species setting used. However, the smallest mean and median differences between the two analyzers were observed when the ADVIA equine setting was used (-0.42 and -0.45 million/µL mean and median differences, respectively). The difference between the equine and Z2 counts ranged from -1.39 to 0.63 million/µL. The mean differences between the 10 fL Z2 count and the ADVIA bovine and goat channel counts were -2.46 and -1.41 million/µL, respectively. 88% of the RBC counts on the ADVIA equine setting were within the TE_a range for the reference method RBC count (i.e. within ±10% of the 10 fL count), while only 28% of the goat setting RBC counts and 0% of the bovine RBC counts were within this TE_a range. The Spearman rank correlation coefficients for the RBC counts measured by the ADVIA were very good ($r \geq 0.92$ for all three species settings), with no evidence of constant bias on any setting. Proportional bias for RBC counts was observed with the ADVIA bovine setting (Table 3).

The reference method MCV was calculated based on a spun PCV and the RBC count from the 10 fL Z2 threshold. This calculated MCV was then compared to the MCV measured by each of the ADVIA species settings. Overall, the MCV values measured by the ADVIA were higher than the calculated MCV, regardless of the species setting used. However, similar to the results for the RBC counts above, the smallest mean and median differences between the two methods were observed with the ADVIA equine measurement (6.8 and 7.6 fL mean and median differences,

respectively). The mean differences between the bovine and goat MCVs and the calculated MCV were 9.5 and 15.6 fL, respectively. The ADVIA measured MCVs were within the TE_a range of the calculated MCV (±7%) in 4% (equine) and 0% (bovine and goat) samples. The correlation coefficients for the ADVIA MCV measurements were fair for the equine and bovine settings (r=0.65 and 0.67, respectively), however there was poor correlation between the MCV on the goat channel and the MCV calculated from the 10 fL Z2 RBC count (MCV=PCV/RBC x 10) (r=0.33). There was no evidence of constant bias for any of the ADVIA settings, however proportional bias was observed with all ADVIA MCV measurements.

It would be impractical to suggest that clinical pathology laboratories use the 10 fL Z2 RBC count to calculate MCV on camelid samples, since a Z2 Coulter counter is not available in all labs. Therefore, a comparison was made between MCV calculated using the Z2 RBC count and the ADVIA equine RBC count. The MCV calculated with the ADVIA equine RBC count was within the TE_a range of the MCV calculated from the Z2 count 84% of the time, with a mean difference of only 1.7 fL. Correlation between these values was good (r=0.70), with no evidence of constant or proportional bias.

Assessment of bias between automated and manual methods

In addition to the 25 EDTA-anticoagulated whole blood samples from the camelid population described above, EDTA whole blood samples were collected from 132 alpacas and llamas in East Tennessee for automated analysis on the ADVIA (equine, bovine, and goat settings), and manual analysis (spun PCV and blood smear review). Results from this patient population were combined with the 25 samples from the population above to assess bias between automated and manual methods for hematocrit, MCHC, and platelet counts.

A spun PCV was considered the gold standard for measurement of volume of the percentage of red cells in blood. Hct is related to PCV, but is determined by the ADVIA using a calculation involving absolute RBC count and measured MCV (Hct = (RBC x MCV)/10). The correlation between the ADVIA Hct and spun PCV was good for all species settings ($r>0.89$), however both constant and proportional bias were observed with all settings. Overall, the ADVIA Hct overestimated the manual PCV by a mean of 3.1% for the bovine setting, 6.5% for the equine setting, and 13.1% for the goat setting (Table 4).

MCHC as reported by the ADVIA is also a calculated value: MCHC = (HGB/Hct) x 100. Since the Hct is calculated by measurements likely to be inaccurate in camelids, and has already been shown to exhibit bias when compared to a spun PCV, bias assessments were also performed between the ADVIA MCHC values and an MCHC calculated using the spun PCV. Hemoglobin concentration (HGB) is measured spectrophotometrically by a chemical reaction after RBC lysis in the ADVIA, and the result was the same for all species settings. Correlation between the ADVIA MCHC and a calculated MCHC was poor for all species settings ($r<0.4$). Constant and proportional bias were observed for the equine and goat settings. Overall, the ADVIA's MCHC values were lower than the calculated MCHC, with the smallest mean difference observed with the bovine setting and the largest difference with the goat setting.

Platelet count and platelet indices are measured by the ADVIA through a combination of size and light scatter measurements. The RBC/PLT reagent forms platelets into "pseudospheres,"[25] and there is no current evidence to suggest that camelid platelets would be resistant to this reaction compared to other species. The platelet component distribution width (PCDW) was

also below 5.5 g/dL for a majority of the EDTA-anticoagulated samples from the first camelid population in this study, regardless of species setting, which has been described as supportive of adequate platelet sphering in dogs.[78] Nonetheless, the ADVIA's automated platelet counts were compared to a manual platelet estimate from a blood smear (average platelets per 1000x field multiplied by 20 = platelet estimate $x10^3/\mu L$), which was recorded for 62 of the camelids in the East Tennessee population. When a range was reported for the platelet estimate, the mid-point of the range was used for comparison analysis. There was no significant difference between the ADVIA's platelet count using the equine setting and a manual platelet estimate ($p=0.152$), although a significant difference was observed between manual estimates and the automated counts from the bovine setting ($p<0.0001$). Due to the low number of samples played back on the goat setting for this population, only 5 paired samples were available for comparison. While the p-value for this comparison was marginally insignificant ($p=0.063$), the lack of overlap in the interquartile ranges for the two methods suggests that the difference in platelet counts between the goat channel and manual estimate may be clinically significant (Figure 7).
No differences in any RBC or platelet values were observed between the ADVIA equine and camelid settings.

Conclusion

The results of this study demonstrate that camelid RBCs do not form isovolumetric spheres in vitro when exposed to the ADVIA RBC/PLT reagent, as do RBCs from other domestic mammal species such as the dog. As a consequence, the methodology used to measure cell volume, identify cells as RBCs, and further characterize the RBCs based on hemoglobin concentration and percentage of cells per volume of blood in automated optical hematology analyzers will likely not provide accurate results for camelids. The differences in RBC counts and indices between

various species settings of the ADVIA, and between the ADVIA and an impedance counter observed in this study support the hypothesis that accurate automated hematology analysis for camelid species is lacking.

The Z2 Coulter counter uses an electrical impedance methodology which does not rely on cell shape, and only counts cells (particles) which fall between user-defined lower and upper volume thresholds. One of the goals of this study was to identify the lower volume threshold that would provide the most accurate RBC counts in camelid blood – i.e. the threshold that includes the most RBCs in its count while excluding the majority of smaller particles (platelets). Based on preliminary research (data not shown), and as might be expected, the Z2 particle count is highest with the lowest volume thresholds (4, 5, and 6 fL). However, examination of the histograms provided by the Z2 counter at these thresholds suggested the possible inclusion of a second cell population, most likely platelets, and therefore a falsely increased RBC count. RBC counts for the 8, 10, and 12 fL thresholds appeared to provide the highest, most accurate cell concentrations, and were therefore investigated further in the current study. Of these three thresholds, the 8 fL threshold provided the highest RBC counts on whole blood and platelet-poor RBC samples, as well as the highest platelet counts for PRP samples. The differences in RBC and platelet counts between the different Z2 thresholds were statistically significant, however evaluation of the overlapping 95% confidence intervals for the means of these counts suggests that the differences may not be clinically significant (Figures 4-6). Use of the 12 fL threshold resulted in the lowest platelet counts, however it was also the only evaluated threshold to provide a lower RBC count than the ADVIA on whole blood samples, suggesting that a proportion of camelid RBCs may be smaller than 12 fL, and be excluded from the total RBC

count. Additionally, when the PRP platelet counts from all three Z2 thresholds were compared to the ADVIA platelet counts, statistically and clinically significant differences were observed, which were greater than the differences between the Z2 thresholds. Given the relatively low concentration of platelets measured by the Z2 counter (tens of thousands per microliter vs. millions per microliter of RBCs), it was suspected that any interference by platelets in whole blood samples at these thresholds would result in minimal artificial increases in the RBC count.

Since the Z2 counter is not a widely used hematology instrument in veterinary laboratories, a second goal of this study was to utilize the results obtained from our Z2 threshold analyses to inform accurate CBC methodology using more common instrumentation. The ADVIA 120/2120 optical hematology analyzers were designed for use with human blood and body fluids, but have been adapted for use with blood and fluids from various domestic mammal species.[23,25,27,31] ADVIA instruments are widely used by veterinary clinical pathology laboratories in both tertiary referral hospitals and commercial laboratories, all of which have the potential to see a large camelid caseload. There is no designated species setting for camelid, camel, alpaca, or llama on the ADVIA, although anecdotal evidence suggests that the ADVIA's equine setting provides the most accurate CBC results for these animals.[28,39] The results of the present study support this claim with regards to the RBC count and thrombogram. When RBC counts from various ADVIA species settings were compared to those measured by or derived from the 10 fL Z2 counts, the ADVIA equine setting resulted in the smallest mean differences, the largest proportion of results within the respective TE_a guidelines, and often the strongest correlations. The ADVIA bovine and goat settings were also included in this study based on the apparent correlation between their RBC volume thresholds and the expected MCV for camelid RBCs, however both of these

settings markedly underestimated the absolute RBC count and overestimated the platelet count. While there is normally some degree of overlap in the RBC and platelet volume ranges regardless of species setting,[25] the observed trends with the bovine and goat settings suggest that a substantial proportion of RBCs may be counted as platelets on these settings. Given that the RBC volume (and hemoglobin concentration) thresholds for all of these ADVIA settings overlap substantially, it is suspected that the ADVIA uses additional criteria for identification and enumeration of RBCs, however it is uncertain what these factors are, or whether they are manageable at the user level.

The linearity of the ADVIA for RBC concentrations above the manufacturer's specified 7,000,000/µL was also an initial concern, given that camelid species can have RBC counts approaching 18,000,000/µL in health. However, the results of the dilution experiment with equine blood in this study indicate that the reportable range of the ADVIA extends to at least 14,115,000 RBCs/µL. There is no evidence to suggest that the analyzer would provide falsely decreased counts at higher RBC concentrations, although the possibility of a deviation from linearity at concentrations higher than those measured here cannot be excluded.

Even with the ADVIA equine setting, there are still automated RBC values which will be inaccurate for camelids as a consequence of their lack of RBC sphering, including MCV, MCHC, and Hct. Therefore, these values should be replaced by calculated MCV, calculated MCHC, and spun PCV. Based on the results of these combined methods and the degree of bias with strictly automated methods (ADVIA), it appears that this combination is needed to provide the most accurate hematology data for camelid species.

One important limitation to this study is that sample analysis was performed at two different institutions, which utilize different instruments and methods to measure the same hematology variables. A spun PCV on the samples from Cornell was measured manually against a standard laminated reader card after centrifugation for 10 minutes in a high-speed microhematocrit centrifuge. Meanwhile, the PCV values from the University of Tennessee were obtained using the "read" function of a low-speed centrifuge after 1 minute of centrifugation. Analysis of the same whole blood sample by both methods will result in slightly different results based on preliminary data using canine blood (data not shown), however this is not expected to impact the conclusions of this study. Additionally, the CBC results from the Cornell camelid population were obtained on an ADVIA 2120, while the majority of the results from the East Tennessee population were obtained on an ADVIA 120. However, there are only minimal differences in the methodologies used by these analyzers to measure the RBC and platelet values of interest to this study,[25] and this would not be expected to significantly affect data interpretation.

Light microscopy was used in the current study to assess the ability of canine and camelid RBCs to undergo a sphering reaction in the ADVIA's RBC/PLT reagent, which was sufficient for support of our hypothesis, but not ideal for truly evaluating three-dimensional sphere formation. Attempts were made in the early stages of this study to obtain scanning electron microscopy (SEM) images of the RBCs in both RBC/PLT reagent and normal saline, however the canine cells had lost their spherical shape in the time between exposure to the reagent and access to the SEM camera, appearing as biconcave discs. Previous work suggests that although the sphering reaction in human RBCs takes place within 1 second with sodium dodecyl sulfate,[75] experimentally sphered RBCs begin to revert to a biconcave disc shape after approximately 8

hours.[79] Since the time between incubation with the sphering reagent and obtaining SEM images in this study exceeded 8 hours, this most likely affected our ability to capture three-dimensional evidence of canine RBC sphere formation. Although the same fixative type and concentration were used in this study as had been previously described in a case of canine elliptocytosis,[8] it seems possible that the more permanent nature of the abnormal RBC shape in that case allowed for retention of the shape for SEM image acquisition, compared to the experimental induction of abnormal RBC shape in the current study.

Finally, although the results of this study show that current automated hematology analysis is inaccurate in camelid species, and provide methodology for more accurate determination of camelid RBC values in a clinical setting, the true values of many of these indices (i.e. MCV) are still unknown. Additional research, potentially involving advanced imaging techniques, is needed to more fully characterize camelid RBCs and our ability to assess them.

CHAPTER II
ENDOPARASITES AND ERYTHROCYTE VALUES

Abstract

Background: *Mycoplasma haemolamae* and gastrointestinal nematodes can cause anemia in camelids. Parasite control programs aim to suppress parasite loads to subclinical levels without excessive deworming that promotes resistance, but there are few evidence-based guidelines for acceptable parasite loads in camelids.

Objectives: In clinically healthy camelids: 1) Compare *M. haemolamae* PCR status and RBC values. 2) Determine the fecal egg count (FEC) threshold above which RBC values are consistently below the median of the reference interval.

Methods: EDTA-anticoagulated blood was collected from 118 clinically healthy, non-anemic adult alpacas and llamas. PCV was measured by centrifugation, RBC and HGB were measured by ADVIA, and *Mycoplasma haemolamae* was detected by real-time PCR. The number of trichostrongyle eggs per gram (epg) was determined in a freshly collected fecal sample using the Modified McMaster's test. Significant differences in RBC values between *M. haemolamae* positive and negative animals, and between various FEC thresholds, were assessed by independent T-test or Mann-Whitney rank sum test.

Results: PCV, HGB, and RBC were not significantly different between *M. haemolamae* positive (N=41) and negative animals ($P>0.5$). PCV, HGB, and RBC were significantly lower in animals with FEC>600epg ($P<0.05$). For 10/10 animals with FEC>600epg, PCV, HGB, and RBC were below the median of the respective reference interval.

Conclusions: Because positive *M. haemolamae* PCR is not associated with lower RBC values in healthy camelids, treatment for *M. haemolamae* may not be necessary in those animals.

FEC>600epg has a negative effect on RBC values, so maintaining FEC below that level is recommended in camelids.

Background

Mycoplasma haemolamae

Mycoplasma haemolamae is a hemotropic bacterial organism (hemoplasma) that is known to cause anemia of variable severity in alpacas and llamas. The mode of transmission of this pathogen is uncertain, although vector-borne transmission is suspected based on studies of similar hemoplasma organisms in other domestic mammals. Iatrogenic, direct horizontal, and vertical modes of transmission have also been proposed.[58,59] Many animals who test positive for this organism never show clinical signs, and it has been suggested that a majority of infections are subclinical, resulting in a carrier state that persists despite antibiotic treatment of infected animals.[62] Anemia is the result of hemolysis, which primarily occurs extravascularly as splenic macrophages remove infected RBCs from circulation. Clinical anemia is reported most often in infected animals who are immunosuppressed, stressed, debilitated, or suffering from a concurrent illness, although organisms can be observed on peripheral blood smears from both ill and clinically healthy animals as epicellular and extracellular ring-shaped structures.[62]

The gold standard test for diagnosis of *M. haemolamae* is a real-time PCR (polymerase chain reaction) assay which is specific for detection of the organism's 16S rRNA gene.[53,54] However, bacteremia in infected animals is transient and cyclical, and it can therefore be difficult to identify *M.haemolamae* as an etiologic agent in cases of anemia.[59] Previous studies have reported that infection with *M. haemolamae* (based on positive real-time PCR results) is not significantly associated with anemia (based on a PCV measurement below the lower reference

limit for the species);[54,56] in one population the mean PCV of PCR-positive animals was higher than that of PCR-negative animals, although this difference was not statistically significant.[56] In another study, one of two *M. haemolamae* positive alpacas was anemic based on PCV and clinical signs, however this individual also had an FEC of 2800 epg, suggesting that the *M. haemolamae* infection may not have been the only cause of anemia, if it was involved in the pathogenesis at all.[61]

Given that PCV reference intervals for alpacas and llamas vary substantially between different laboratories, and that PCV is only one index of RBC mass, one goal of this study was to perform a more comprehensive assessment of the relationship between *M. haemolamae* PCR status and RBC values (including PCV, RBC count and HGB concentration). The hypothesis was that clinically healthy animals who are positive for *M. haemolamae* on real-time PCR would have significantly lower RBC value(s) compared to clinically healthy animals with negative PCR results. If such a relationship exists, it might indicate a negative impact of *M. haemolamae* infection despite the lack of anemia or clinical illness, potentially explaining the increased susceptibility of infected animals to the development of clinical anemia in the presence of concurrent illness (e.g. heavy gastrointestinal nematode burdens) or immunosuppression.

Gastrointestinal nematodes

Gastrointestinal nematode infections are also a common cause of anemia in New World camelids. *Haemonchus contortus*, a trichostrongylid nematode residing in the third gastric compartment (C3), is the most common and clinically significant endoparasite of alpacas and llamas. *H. contortus* is also of clinical importance in small ruminants, in whom the parasite resides in the abomasum.[66] Anemia is the result of blood loss, both through direct feeding of

the adult worm on the host's blood supply, as well as irritation of the gastric mucosa.[66] Trichostrongylid infections can be diagnosed and semi-quantified with the fecal egg count test (FEC), which measures the number of trichostrongyle eggs per gram (epg) of feces. However, this test cannot differentiate *H. contortus* eggs from other trichostrongyle eggs of similar size and morphology (e.g. *Teladorsagia circumcincta, Trichostrongylus axei*, etc).[66] In addition, there are no established thresholds for defining clinically significant FECs in alpacas and llamas. In one study performed in the southeastern United States, a majority of camelids with clinical anemia had an FEC >1000 epg,[72] while a separate study in a population of alpacas from New Zealand identified 250 epg as a "high and clinically significant" egg count.[61] This wide range of potentially significant values makes it difficult to base treatment decisions on FEC alone.

The FAMACHA system (Faffa Malan Chart, named for its inventor, Dr. Francois "Faffa" Malan) has been validated as a clinical tool for assessing the degree of anemia due to *H. contortus* infection in South American camelids.[72] In this system, an animal's conjunctival mucous membrane color is evaluated side-by-side with a card showing varying shades of pink. A score of 1 corresponds to bright pink/red membranes (normal, healthy) and a score of 5 corresponds to white membranes (severe anemia). Deworming treatment is typically recommended for animals with scores of 3 or higher.

FEC has been shown to be negatively correlated to PCV and other RBC values in clinically ill alpacas and llamas, and in naturally and experimentally infected sheep. [66,73,74] Additionally, experimental infection of sheep with *Haemonchus sp.* demonstrated a linear relationship between adult worm numbers, fecal egg counts, and blood loss.[70] However, there has been no

published documentation of any correlation between FEC and RBC values in clinically healthy camelids. Additionally, currently available tools used by practitioners and owners to identify animals in need of deworming treatment, such as the FAMACHA system, are only capable of identifying animals who are already anemic, potentially missing animals with high nematode burdens who are not yet clinically ill. Conversely, deworming all animals in a herd regardless of their potential nematode burden is expensive and unnecessary, and promotes parasite resistance to deworming medications. A second objective of this study was therefore to assess the relationship between FEC and RBC values (PCV, RBC, and HGB) in a population of clinically healthy (non-anemic) adult camelids, and to identify an FEC threshold above which RBC values are consistently (>50% of the time) below the median of their respective reference intervals (i.e. what is the FEC threshold at which a negative impact on RBC values is consistently observed?). Identification of this FEC threshold would provide additional guidance to owners and veterinarians in determining which animals could potentially benefit from deworming treatment, prior to the development of clinical anemia.

Materials and Methods

Patient population and sample collection

Venous whole blood was collected from clinically healthy adult (≥ 1-year-old) alpacas and llamas from 12 farms in eastern Tennessee between July and October 2015. Health was defined as a lack of significant abnormalities on physical examination as determined by an experienced veterinarian, a FAMACHA score of 3/5 or lower,[72] a body condition score (BCS) of 2.5/5 or higher, and no history of illness or injury within the previous 3 months. All examinations and

sample collections took place on the farms, and informed client consent was obtained for all procedures (approved UT IACUC protocol 2298-0914).

Samples from a maximum of 12 animals were collected per farm. Individual and herd histories were obtained, including species, age, sex, pregnancy/breeding status, housing, diet, vaccines, and parasite control practices. After examination of each animal, a maximum of 10 mL of blood was collected by an experienced veterinarian from an external jugular vein using a 12 mL plastic syringe (Covidien LLC, Mansfield, MA) attached to a 20 gauge, 1.5-inch needle (Covidien LLC, Mansfield, MA). The blood was placed in a 4 mL K_2EDTA tube (Becton Dickinson, Franklin Lakes, NJ) and transported to the University of Tennessee clinical pathology laboratory in a cooler that was maintained at 4^{o}C until arrival. Blood samples were promptly analyzed (all analysis was performed within 6 hours of sample collection) by trained laboratory personnel. Samples were evaluated for appropriate filling of the EDTA tube, and for clot formation and/or gross lipemia. Underfilled, clotted, and lipemic samples were excluded. A CBC with reticulocyte count was performed on each sample using the equine setting of the ADVIA 120 hematology instrument according to laboratory standard operating procedures (SOP).

Manual PCVs were determined following one minute of centrifugation in a rapid fixed angle head microhematocrit centrifuge (HemataStat II, EKF Diagnostics, Boerne, TX). The centrifuge's "read" function was used to determine the PCV; this function is calibrated for the microhematocrit's centrifuge speed. Proper microhematocrit centrifuge function was verified using an electronic tachometer prior to beginning the study, and a series of test centrifugations of camelid blood (data not shown) confirmed there is no significant difference in PCV with

centrifugation times longer than one minute. ADVIA 120 and manual PCV analyses were performed by a licensed medical technologist trained in instrument use and following laboratory SOP. The ADVIA 120 underwent daily quality control using 3-level QCM (OPTIpoint, Siemens Healthcare Diagnostics, Tarrytown, NY).

A minimum of one blood smear was prepared from each sample, and reviewed by a medical technologist according to laboratory SOP. Blood smear review included a leukocyte differential, RBC morphology review, and a manual platelet estimate. Blood smears were also reviewed by a board-certified veterinary clinical pathologist.

A fresh fecal sample was also collected digitally from the rectum of each animal by trained personnel, placed in an individual, clean plastic bag, and transported in a cooler to the UTVMC parasitology laboratory.

M. haemolamae and FEC testing

After CBC analysis, the remaining EDTA-anticoagulated whole blood from each animal was stored at -20^{o}C for up to 30 days. DNA extraction and real-time PCR for *Mycoplasma haemolamae* were performed, using previously described methods[54] (Applied Biosystems StepOne™, ThermoFisher Scientific, Waltham, MA). Positive and negative control samples were included in each run. Individual samples were classified as positive or negative based on their C_T (cycle threshold) value, with samples having a C_T value less than or equal to 35.0 classified as positive.

Fecal egg counts were performed on each fecal sample using the Modified McMaster's test, as has been previously described for alpacas and llamas.[67] Briefly, 2 grams of feces were mixed with 28 mL of a sodium nitrate flotation solution with a specific gravity of 1.200. The mixture was loaded into a McMaster's slide, and examined under light microscopy after 5 minutes of flotation. The number of strongylid eggs in both chambers was counted at 100x total magnification, and the total number counted in each sample was multiplied by 50 to obtain results in eggs per gram of feces (epg). An egg count of 0 was reported as <50 epg. Since FECs are based on visual identification of parasite eggs, it is not possible to distinguish between the various species of strongylid eggs with this method. Therefore, nematode larval cultures were also performed on a subset of the fecal samples in order to further classify the strongylid species present.

Statistical analysis

Statistical analysis was performed using commercial software (MedCalc Statistical Software version 17.4 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2017). Animals were classified as anemic or non-anemic based on having a PCV, RBC count, or HGB concentration below or above the lower limit, respectively, for the appropriate laboratory reference interval. The sample population was also divided into two groups based on *M. haemolamae* PCR status: *M. haemolamae*-positive (Mhl-positive) and *M. haemolamae*-negative (Mhl-negative). The distributions of RBC values (PCV, RBC, HGB) in both groups were assessed for normality using the D'Agostino-Pearson test. The means/medians of the two groups were compared using the independent T-test or Mann-Whitney rank sum test. Significance was based on a p-value <0.05 and lack of overlap in 95% confidence intervals for the mean/median. The sample population was also stratified into groups based on FEC: animals with FEC<450 epg,

600<FEC<1000 epg, and FEC≥1000 epg. RBC values were compared between the different FEC strata using a one-way ANOVA or Kruskal-Wallis test, and significance was based on a p-value<0.05 and lack of overlap in 95% confidence intervals for the mean/median. Comparison of RBC values was also performed between Mhl-positive and Mhl-negative animals in the different FEC strata. Receiver operator characteristic (ROC) curves were used to identify an FEC threshold with optimal sensitivity and specificity for predicting a negative effect on RBC values.

Results

Animals

EDTA-anticoagulated whole blood and fecal samples were collected from 118 clinically healthy adult New World camelids. There were 98 alpacas (55 females, 39 intact males, and 4 castrated males) and 20 llamas (13 females, 1 intact male, and 6 castrated males) in the study population. The alpacas ranged in age from 1-15 years (mean = 5.42 years), and the llamas ranged in age from 3-16 years (mean = 11 years). There were 10 alpacas (3 females and 7 males) whose ages were unknown or not recorded. Four animals were mildly anemic based on a PCV (n=2) or HGB concentration (n=2) below the lower limit of the laboratory reference interval (20% and 7.5 g/dL, respectively); however, these animals were not clinically ill and had FAMACHA scores > 3 due to study inclusion criteria. RBC counts were within the reference interval for all animals, and PCV and HGB concentration were within their respective reference intervals for the remaining 114/118 animals. A total of 41/118 animals (37 alpacas and 4 llamas) in this study population tested positive for *M. haemolamae* on real-time PCR, resulting in a 34.7% prevalence. No *Mycoplasma* organisms were observed on blood smears from any animal. FECs in this population ranged from <50 to 3200 epg, with a median and standard deviation of 100 ±

50 epg. In addition to strongylid eggs, other nematode eggs (*Nematodirus* spp*., Aonchotheca (Capillaria)* spp.), coccidia (*Eimeria* spp.), and tapeworm eggs (*Moniezia spp*.) were noted in the McMaster chamber after fecal flotation; their presence was described qualitatively. Larval cultures were performed on 18/118 fecal samples, and the L3 stage of *H. contortus* grew in all cultures. Additional culture findings included *Trichostrongylus* spp. (n=4) and *Nematodirus* spp*.* (n=4).

M. haemolamae and RBC values

RBC values for Mhl-positive animals were consistently lower than those for Mhl-negative animals, however the differences between the group means for each value (PCV, RBC count, and HGB concentration) were not statistically significant. The mean PCV of the Mhl-positive animals (n=41) was 32.1%, and the mean PCV of the Mhl-negative group (n=77) was 32.8% (p=0.526). The mean RBC count for the Mhl-positive group was 12.01 million/µL; the mean for the Mhl-negative group was 12.02 million/µL (p=0.981). The mean HGB concentration for the Mhl-positive group was 12.76 g/dL, and the mean concentration for the Mhl-negative group was 12.92 g/dL (p=0.725) (Figure 8). Mean RBC values (PCV, RBC count, HGB) between Mhl-positive and Mhl-negative animals with an FEC>600 epg, and between Mhl-positive animals with C_T values less than 30.0 and Mhl-negative animals were also compared, with no statistically significant differences observed (Figure 9).

Fecal egg count and RBC values

RBC values for animals in different FEC strata (<450 epg, between 600 and 1000 epg, and ≥1000 epg) were compared. A one-way ANOVA was used to compare the RBC counts between the strata. The distribution of PCV and HGB values in the various strata were non-parametric, and the Kruskal-Wallis test was used for these comparisons. The mean RBC counts for the different

FEC strata were 12.3 million/µL (<450 epg), 10.65 million/µL (600<FEC<1000 epg), and 9.70 million/µL (≥1000 epg). There was a significant difference between the lowest and highest strata (p=0.009), however neither of these two strata was significantly different from the middle stratum. The median HGB concentrations for the different strata were 13.2 g/dL, 12.0 g/dL, and 9.9 g/dL, respectively. Similar to the findings for RBC count, there was a significant difference between the lower and upper strata (p=0.0075); the middle stratum was not significantly different from either the lower or upper strata. The median PCVs for the different strata were 33.5%, 30.0%, and 28.0%. There were no significant difference between the PCVs of the various strata (p=0.105) (Figure 10). Manual platelet estimates and refractometric plasma total protein concentrations were also compared between animals with an FEC <600 epg and those with an FEC >600 epg. There were no statistically significant differences between platelet counts or total protein concentrations in animals with low vs. high FECs (data not shown).

In concordance with the trend observed above of decreasing RBC values with increasing FECs, a scatterplot of HGB concentration vs. FEC revealed that all animals with an FEC greater than 600 epg had a HGB concentration below the median of the camelid reference interval (Figure 11). This trend was also observed for RBC count and PCV in animals with an FEC greater than 600 epg.

Receiver operator characteristic (ROC) curves were used to identify the FEC threshold with the optimal combination of sensitivity and specificity for predicting RBC values below the median of their respective reference intervals. At a FEC threshold of >600 epg, the sensitivity for a HGB concentration <13.2 g/dL was 100%. Similarly, the sensitivity for a PCV below 33.5% was 100%

at an FEC threshold >600 epg. The specificity for these HGB concentrations and PCV values at this threshold were 49.1% and 45.3%, respectively (Figure 12). The specificity did not change substantially for either HGB or PCV with the use of different FEC thresholds (450, 700, or 1000 epg). The sensitivity for HGB and PCV was 82.4% and 85.4%, respectively, at a threshold of 450 epg, and 100% at the 700 and 1000 epg thresholds.

Conclusion

This study found no statistically significant differences in mean RBC values (PCV, HGB, RBC count) between *Mycoplasma haemolamae* positive and negative animals from a population of clinically healthy, adult alpacas and llamas. This lack of relationship between RBC values and *M. haemolamae* PCR status was independent of both the degree of *M. haemolamae* positivity (based on C_T value) and of fecal egg count. The results of this study support the findings of previous research, suggesting that the majority of cases of *M. haemolamae* infections are subclinical and that antimicrobial treatment is likely not warranted based on PCR status alone.[56,62] However, our hypothesis that there would be a negative correlation between *M. haemolamae* PCR status and RBC values in clinically healthy adult camelids was rejected. The presence of such a correlation would have suggested that even in a subclinical state, the bacteria could have a negative impact on the animal's RBC mass and/or oxygen-carrying ability, thereby increasing susceptibility to the development of clinical anemia in the presence of concurrent disease (i.e. high gastrointestinal nematode burden, malnutrition, etc).

The prevalence of *M. haemolamae* infection in our study population (34.7%) is higher than that reported in previous prevalence studies for this organism in other geographic areas. [56,60,61] This increased prevalence may reflect the subtropical latitude at which the current population

resides (possibly supporting vector-borne disease transmission), or different husbandry practices for camelids in various parts of the world. The average number of Mhl-positive animals per farm in this study was 3, however the proportion of affected animals on a given farm ranged from 0-100%, suggesting the influence of different husbandry practices on disease transmission and prevalence. Given the relatively high prevalence of *M. haemolamae* infection and the overall size of the sample population, it is unlikely that rejection of our hypothesis was the result of insufficient statistical power. However, the lack of relationship with RBC values observed in this study may reflect the exclusion of animals with high FAMACHA scores and/or low body condition scores. Even though separation of Mhl-positive animals based on C_T value did not demonstrate significant differences in RBC values compared to Mhl-negative animals, it is possible that use of a quantitative PCR assay (instead of the semi-quantitative assay used in the current study) may have allowed for increased sensitivity in detection of changes in RBC mass in animals with higher DNA copy numbers. Additionally, the exclusion of alpacas and llamas less than 1 year old from the current study precluded evaluation of the potential effects of M. haemolamae infection on RBC values in young animals. Given that cases of clinical anemia have been reported in young, potentially immunocompromised camelids,[52,59] the possibility that negative RBC effects may be more likely to be observed in association with infection in animals less than 1 year old should be considered for future study. Ultimately, further study is also needed to more fully comprehend the subclinical nature of *M. haemolamae* infections in adult camelids, and the factors which allow for the development of clinical hemolytic anemia.

The results of the present study do demonstrate a trend of decreasing RBC values with increasing FECs, and that clinically healthy animals with high FECs (>1000 epg) are statistically more likely to have lower RBC counts and HGB concentration than animals with FECs <450 epg. Additionally, an FEC threshold of 600 epg has excellent sensitivity and fair specificity, in this population of clinically healthy camelids, for predicting a PCV and HGB concentration below the median of the reference interval. Alpaca and llama owners and veterinarians currently have limited tools available for assessing the clinical significance of gastrointestinal parasite burdens and the need for parasite treatment. The goal of deworming is to prevent and/or treat the effects of clinical blood loss anemia in at-risk animals, while avoiding the promotion of parasite resistance to the anthelminthic medications. However, owners and veterinarians are often faced with the limited options of either deworming clinically anemic animals (as identified by a FAMACHA score),[72] or prophylactically deworming all animals on the farm (or a random subset) in hopes of reaching those animals who are most likely to develop clinical disease and/or shed large numbers of eggs. The identification of a FEC threshold above which RBC values are likely to be negatively affected should therefore aid substantially in making treatment decisions, allowing for targeted deworming of those animals with clinically significant nematode burdens. However, FEC testing via the Modified McMaster's test is inherently of low analytical sensitivity, as each strongylid egg counted is interpreted as representing 50 eggs. The lowest obtainable FEC is therefore <50 epg, and all egg counts are expressed in intervals of 50; this test is therefore only semi-quantitative. FEC testing is also relatively imprecise, since counts can vary substantially between observers, between different portions of the same fecal sample, and with variations in the concentration of the flotation solution, sample dilution, and volume of the counting chamber.[69] Additionally, the correlation between trichostrongylid egg numbers in

feces and adult worm burden of a particular species (i.e. *Haemonchus* spp.) in the GI tract is uncertain. The FEC test as performed in this study is relatively simple in its methodology, and has the potential to be replicated on-farm by alpaca and llama owners; however, additional research assessing the precision and accuracy of owner-performed FECs compared to those performed by diagnostic laboratory personnel would be required prior to implementation of the use of these diagnostic guidelines for animal owners. Additionally, the present study only allowed for assessment of the association between FEC and RBC values at a single point in time. It would therefore be useful to perform a prospective cohort study in the future, in order to determine whether an FEC over 600 epg is truly predictive for the development of anemia. The inclusion of young camelids (less than 1 year old) in any future studies would also be helpful in assessing the effects of gastrointestinal nematodes on RBC values in this age group.

An important limitation of both parts of this study is the inability to exclude the effects of other factors (internal and external) on the measured RBC values. RBC values are analyzed as a reflection of an animal's overall RBC mass, which is affected by multiple factors including age, hydration status, nutrient balance, and renal and bone marrow function. Despite the presence of known causes of anemia (*Mycoplasma* spp., gastrointestinal nematodes), it is difficult to attribute changes in RBC values (or lack thereof) solely to the presence or absence of these organisms. For example, decreased RBC values may reflect iron deficiency, or the effects of inflammation on erythropoiesis, rather than direct blood loss from endoparasites.

In conclusion, the results of the present study confirm the findings of previous studies which demonstrate a lack of association between *M. haemolamae* infection and PCV in alpacas and llamas, and expand these findings to also include a lack of association with HGB concentration or RBC count. Overall, this suggests that treatment of clinically healthy *M. haemolamae* PCR-positive animals is not warranted, although *M. haemolamae* infection cannot be ruled out as a contributing cause to the development of clinical anemia in these camelids. Finally, with additional research and method validation, the use of an FEC threshold of <600 epg in clinically healthy alpacas and llamas may provide one component for guiding deworming strategies.

CONCLUSION

The present studies provide, to the author's knowledge, the first report of validated hematology methods for camelid species, as well as the first set of evidence supporting the existence of an acceptable endoparasite load in camelids. Camelid erythrocytes do not sphere when mixed with ADVIA sphering reagent, making some RBC values inaccurate. Based on our results, the recommended method for reporting the erythrogram and thrombogram from camelids using the ADVIA 120 is as follows:

- RBC count: ADVIA equine setting
- HGB concentration: ADVIA equine setting
- Volume percentage of RBCs in whole blood: PCV
- MCV: Calculated (PCV divided by ADVIA equine setting RBC count multiplied by 10)
- MCHC: Calculated (ADVIA equine setting HGB divided by PCV multiplied by 100)
- Platelet count: ADVIA equine setting

RBC values are not significantly different between *M. haemolamae* positive and negative animals, but are significantly lower in animals with FEC>600epg. For all animals with FEC>600epg, RBC values are below the medians of the reference intervals. Positive *M. haemolamae* PCR is not associated with lower RBC values in healthy camelids, consistent with previous reports that most infections are subclinical. Maintaining FEC below 600 epg is recommended in camelids.

REFERENCES

1. Tornquist SJ. Clinical pathology of llamas and alpacas. *Vet Clin North Am Food Anim Pract.* 2009;25(2):311-322.
2. Tornquist SJ, Cebra C. Clinical Pathology. In: *Llama and Alpaca Care.*2014:338-350.
3. Vap L, Bohn AA. Hematology of camelids. *Vet Clin North Am Exot Anim Pract.* 2015;18(1):41-49.
4. Raskin R, Wardrop K. Species specific hematology. In. *Schalm's Veterinary Hematology, 6th edition, Wiley-Blackwell, Iowa*2010:797-1018.
5. Omorphos S, Hawkey CM, Rice-Evans C. The elliptocyte: a study of the relationship between cell shape and membrane structure using the camelid erythrocyte as a model. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry.* 1989;94(4):789-795.
6. Harvey JW. Chapter 7 - The Erythrocyte: Physiology, Metabolism, and Biochemical Disorders. In: *Clinical Biochemistry of Domestic Animals (Sixth Edition).* San Diego: Academic Press; 2008:173-240.
7. Silveira P, Cynober T, Dhermy D, Mohandas N, Tchernia G. Red blood cell abnormalities in hereditary elliptocytosis and their relevance to variable clinical expression. *American Journal of Clinical Pathology.* 1997;108(4):391-399.
8. Di Terlizzi R, Gallagher PG, Mohandas N, et al. Canine elliptocytosis due to a mutant β-spectrin. *Veterinary Clinical Pathology.* 2009;38(1):52-58.
9. Gulliver G. *Observations on the Sizes and Shapes of the Red Corpuscles of the Blood of Vertebrates.* 1875.
10. Jain N, Keeton K. Morphology of camel and llama erythrocytes as viewed with the scanning electron microscope. *British Veterinary Journal.* 1974;130(3):288-291.
11. Rana J, Bhardwaj R, Singh R. Morphological studies of erythrocytes in different animal species. *INDIAN VETERINARY JOURNAL.* 1992;69(6):505-507.
12. Lewis JH. Comparative hematology—studies on camelidae. *Comparative Biochemistry and Physiology Part A: Physiology.* 1976;55(4):367-371.
13. Bogner P, Miseta A, Berente Z, Schwarcz A, Kotek G, Repa I. Osmotic and diffusive properties of intracellular water in camel erythrocytes: effect of hemoglobin crowdedness. *Cell Biol Int.* 2005;29(9):731-736.
14. Bogner P, Csutora P, Cameron IL, Wheatley DN, Miseta A. Augmented water binding and low cellular water content in erythrocytes of camel and camelids. *Biophysical Journal.* 1998;75(6):3085-3091.
15. Jain N. MEASUREMENT, INTERPRETATION, FACTORS INVOLVED, AND MECHANISM OF OSMOTIC FRAGILITY WITH OBSERVATIONS ON ANIMAL ERYTHROCYTES. *Veterinary Clinical Pathology.* 1972;1(3):3-24.
16. Schroter R, Filali RZ, Brain A, Jeffrey P, Robertshaw D. Influence of dehydration and watering on camel red cell size: a scanning electron microscopic study. *Respiration Physiology.* 1990;81(3):381-390.
17. Eitan A, Aloni B, Livne A. Unique properties of the camel erythrocyte membrane: II. Organization of membrane proteins. *Biochimica et Biophysica Acta (BBA)-Biomembranes.* 1976;426(4):647-658.
18. Smith JE, Mohandas N, Shohet SB. Variability in erythrocyte deformability among various mammals. *American Journal of Physiology-Heart and Circulatory Physiology.* 1979;236(5):H725-H730.

19. McPherson RA, Sawyer WH, Tilley L. Band 3 mobility in camelid elliptocytes: implications for erythrocyte shape. *Biochemistry.* 1993;32(26):6696-6702.
20. Khodadad JK, Weinstein RS. The band 3-rich membrane of llama erythrocytes: studies on cell shape and the organization of membrane proteins. *Journal of Membrane Biology.* 1983;72(3):161-171.
21. Poyart C, Wajcman H, Kister J. Molecular adaptation of hemoglobin function in mammals. *Respiration Physiology.* 1992;90(1):3-17.
22. Bartels H, Hilpert P, Barbey K, et al. Respiratory functions of blood of the yak, llama, camel, Dybowski deer, and African elephant. *American Journal of Physiology--Legacy Content.* 1963;205(2):331-336.
23. Moritz A, Becker M. Automated hematology systems. In. *Schalm's Veterinary Hematology. 6th edn. Edited by Weiss DJ, Wardrop KJ: Ames, Iowa: Wiley-Blackwell*2010:1054-1056.
24. Tycko D, Metz M, Epstein E, Grinbaum A. Flow-cytometric light scattering measurement of red blood cell volume and hemoglobin concentration. *Applied Optics.* 1985;24(9):1355-1365.
25. Harris N, Kunicka J, Kratz A. The ADVIA 2120 hematology system: flow cytometry-based analysis of blood and body fluids in the routine hematology laboratory. *Laboratory Hematology: Official Publication of the International Society for Laboratory Hematology.* 2005;11(1):47-61.
26. Perez-Ecija A, Gonzalez-De Cara CA, Aguilera-Aguilera R, Estepa JC, Rubio MD, Mendoza FJ. Comparison of donkey hemogram using the LaserCyte hematology analyzer, an impedance system, and a manual method. *Vet Clin Pathol.* 2014;43(4):525-537.
27. Giordano A, Rossi G, Pieralisi C, Paltrinieri S. Evaluation of equine hemograms using the ADVIA 120 as compared with an impedance counter and manual differential count. *Veterinary Clinical Pathology.* 2008;37(1):21-30.
28. Dawson DR, DeFrancisco RJ, Stokol T. Reference intervals for hematologic and coagulation tests in adult alpacas (Vicugna pacos). *Vet Clin Pathol.* 2011;40(4):504-512.
29. Weiser M, Fettman M, Van Houten D, Johnson L, Garry F. Characterization of erythrocytic indices and serum iron values in healthy llamas. *American Journal of Veterinary Research.* 1992;53(10):1776-1779.
30. Hajduk P. Haematological reference values for alpacas. *Australian Veterinary Journal.* 1992;69(4):89-90.
31. Siemens. Advia 120 Hematology System, Performance Specifications. https://www.healthcare.siemens.com/hematology/systems/advia-120-hematology-system/technical-specifications. Accessed January 3, 2018.
32. Siemens. Module 4: ADVIA 120 Methods.
33. Latimer P. Light scattering by ellipsoids. *Journal of Colloid and Interface Science.* 1975;53(1):102-109.
34. Kinnunen M, Kauppila A, Karmenyan A, Myllylä R. Effect of the size and shape of a red blood cell on elastic light scattering properties at the single-cell level. *Biomedical Optics Express.* 2011;2(7):1803-1814.
35. Borovoi AG, Naats EI, Oppel UG. Scattering of light by a red blood cell. *Journal of Biomedical Optics.* 1998;3(3):364-373.

36. Mohandas N, Kim Y, Tycko D, Orlik J, Wyatt J, Groner W. Accurate and independent measurement of volume and hemoglobin concentration of individual red cells by laser light scattering. *Blood.* 1986;68(2):506-513.
37. Mohandas N, Clark MR, Kissinger S, Bayer C, Shohet SB. Inaccuracies associated with the automated measurement of mean cell hemoglobin concentration in dehydrated cells. *Blood.* 1980;56(1):125-128.
38. Rice-Evans C, Omorphos S, White JM. The effects of hydrazine on sickle cells. *Biochimica et Biophysica Acta (BBA)-Biomembranes.* 1982;691(2):367-371.
39. Dawson Soto DR, Stokol T, DeFrancisco R. Clarification of methods for hematologic reference intervals in adult alpacas. *Veterinary Clinical Pathology.* 2014;43(1):1-1.
40. Pan L-L, Chen C-M, Huang W-T, Sun C-K. Enhanced Accuracy of Optical Platelet Counts in Microcytic Anemia. *Laboratory Medicine.* 2014;45(1):32-36.
41. Grimes CN, Fry MM. Customization of Advia 120 thresholds for canine erythrocyte volume and hemoglobin concentration, and effects on morphology flagging results. *The Canadian Veterinary Journal.* 2014;55(12):1173.
42. Beckman Coulter, Inc. Z Series Coulter counter: cell counting and sizing. Fullerton, CA. 2004.
43. England JM. Reference method for the enumeration of erythrocytes and leucocytes. *Clinical & Laboratory Haematology.* 1994;16(2):131-138.
44. Chernyshev AV, Tarasov PA, Semianov KA, Nekrasov VM, Hoekstra AG, Maltsev VP. Erythrocyte lysis in isotonic solution of ammonium chloride: Theoretical modeling and experimental verification. *Journal of Theoretical Biology.* 2008;251(1):93-107.
45. Johnson J, Edmondson M, Walz P, Marley M, Givens M. Comparison of clinical, hematological, and virological findings in alpacas (Lama pacos) inoculated with bovine viral diarrhea virus isolates of alpaca or bovine origin. *Small Ruminant Research.* 2010;94(1):66-72.
46. Topliff CL, Smith DR, Clowser SL, et al. Prevalence of bovine viral diarrhea virus infections in alpacas in the United States. *Journal of the American Veterinary Medical Association.* 2009;234(4):519-529.
47. Abdurahman OAS, Saad AM. Characterization of camel leukocytes by flow cytometry and microscopic evaluation of granulocyte phagocytosis of fluorescent bacteria. *Transboundary and Emerging Diseases.* 1996;43(1-10):111-117.
48. Killeen AA, Long T, Souers R, Styer P, Ventura CB, Klee GG. Verifying performance characteristics of quantitative analytical systems: calibration verification, linearity, and analytical measurement range. *Archives of Pathology & Laboratory Medicine.* 2014;138(9):1173-1181.
49. Serebrennikova YM, Huffman DE, Garcia-Rubio LH. Characterization of red blood cells with multiwavelength transmission spectroscopy. *Biomed Res Int.* 2015;2015:382641.
50. Tomaiuolo G, Rossi D, Caserta S, Cesarelli M, Guido S. Comparison of two flow-based imaging methods to measure individual red blood cell area and volume. *Cytometry A.* 2012;81(12):1040-1047.
51. Messick JB. Hemotrophic mycoplasmas (hemoplasmas): a review and new insights into pathogenic potential. *Veterinary Clinical Pathology.* 2004;33(1):2-13.
52. Allison R, Meinkoth J. Anemia caused by rickettsia, mycoplasma, and protozoa. *Schalm's Veterinary Hematology 6th Ed Wiley-Blackwell.* 2010.

53. Guimaraes AM, Toth B, Santos AP, do Nascimento NC, Kritchevsky JE, Messick JB. Genome sequence of “Candidatus Mycoplasma haemolamae” strain purdue, a red blood cell pathogen of alpacas (Vicugna pacos) and llamas (Lama glama). *Journal of Bacteriology.* 2012;194(22):6312-6313.
54. Meli ML, Kaufmann C, Zanolari P, et al. Development and application of a real-time TaqMan((R)) qPCR assay for detection and quantification of 'Candidatus Mycoplasma haemolamae' in South American camelids. *Vet Microbiol.* 2010;146(3-4):290-294.
55. Ghazisaeedi F, Atyabi N, Zahrai Salehi T, et al. A molecular study of hemotropic mycoplasmas (hemoplasmas) in cats in Iran. *Vet Clin Pathol.* 2014;43(3):381-386.
56. Tornquist SJ, Boeder L, Rios-Phillips C, Alarcon V. Prevalence of Mycoplasma haemolamae infection in Peruvian and Chilean llamas and alpacas. *Journal of Veterinary Diagnostic Investigation.* 2010;22(5):766-769.
57. Reagan W, Garry F, Thrall M, Colgan S, Hutchison J, Weiser M. The clinicopathologic, light, and scanning electron microscopic features of eperythrozoonosis in four naturally infected llamas. *Veterinary Pathology.* 1990;27(6):426-431.
58. Fisher DJ, Zinkl JG. Eperythrozoonosis in a One-Day-Old Llama. *Veterinary Clinical Pathology.* 1996;25(3):93-94.
59. Almy FS, Ladd SM, Sponenberg DP, Crisman MV, Messick JB. Mycoplasma haemolamae infection in a 4-day-old cria: support for in utero transmission by use of a polymerase chain reaction assay. *The Canadian Veterinary Journal.* 2006;47(3):229.
60. Crosse P, Ayling R, Whitehead C, et al. First detection of 'Candidatus Mycoplasma haemolamae' infection in alpacas in England. *Vet Rec.* 2012;171(3):71.
61. Dittmer KE, Hinkson JA, Dwyer C, Adlington B, van Andel M. Prevalence of Candidatus Mycoplasma haemolamae, bovine viral diarrhoea virus, and gastrointestinal parasitism in a sample of adult New Zealand alpaca (Vicugna pacos). *N Z Vet J.* 2018;66(1):9-15.
62. Tornquist SJ, Boeder LJ, Cebra CK, Messick J. Use of a polymerase chain reaction assay to study response to oxytetracycline treatment in experimental Candidatus Mycoplasma haemolamae infection in alpacas. *American Journal of Veterinary Research.* 2009;70(9):1102-1107.
63. Ballweber LR. Ecto- and endoparasites of new world camelids. *Vet Clin North Am Food Anim Pract.* 2009;25(2):295-310.
64. Cafrune MaM, Aguirre DH, Rickard LG. First report of Lamanema chavezi (Nematoda: Trichostrongyloidea) in llamas (Lama glama) from Argentina. *Veterinary Parasitology.* 2001;97(2):165-168.
65. Dargie J, Allonby E. Pathophysiology of single and challenge infections of Haemonchus contortus in Merino sheep: Studies on red cell kinetics and the “self-cure” phenomenon. *International Journal for Parasitology.* 1975;5(2):147-157.
66. Edwards EE, Garner BC, Williamson LH, Storey BE, Sakamoto K. Pathology of Haemonchus contortus in New World camelids in the southeastern United States: a retrospective review. *J Vet Diagn Invest.* 2016;28(2):105-109.
67. Cebra CK, Stang BV. Comparison of methods to detect gastrointestinal parasites in llamas and alpacas. *Journal of the American Veterinary Medical Association.* 2008;232(5):733-741.
68. Galvan N, Middleton JR, Nagy DW, Schultz LG, Schaeffer JW. Anthelmintic resistance in a herd of alpacas (Vicugna pacos). *The Canadian Veterinary Journal.* 2012;53(12):1310.

69. Cringoli G, Rinaldi L, Veneziano V, Capelli G, Scala A. The influence of flotation solution, sample dilution and the choice of McMaster slide area (volume) on the reliability of the McMaster technique in estimating the faecal egg counts of gastrointestinal strongyles and Dicrocoelium dendriticum in sheep. *Vet Parasitol.* 2004;123(1-2):121-131.
70. Le Jambre L. Relationship of blood loss to worm numbers, biomass and egg production in Haemonchus infected sheep. *International Journal for Parasitology.* 1995;25(3):269-273.
71. Burke JM, Kaplan RM, Miller JE, et al. Accuracy of the FAMACHA system for on-farm use by sheep and goat producers in the southeastern United States. *Vet Parasitol.* 2007;147(1-2):89-95.
72. Storey BE, Williamson LH, Howell SB, et al. Validation of the FAMACHA(c) system in South American camelids. *Vet Parasitol.* 2017;243:85-91.
73. Di Loria A, Veneziano V, Piantedosi D, et al. Evaluation of the FAMACHA system for detecting the severity of anaemia in sheep from southern Italy. *Vet Parasitol.* 2009;161(1-2):53-59.
74. Vanimisetti H, Andrew S, Zajac A, Notter D. Inheritance of fecal egg count and packed cell volume and their relationship with production traits in sheep infected with. *Journal of Animal Science.* 2004;82(6):1602-1611.
75. Kim YR, Ornstein L. Isovolumetric sphering of erythrocytes for more accurate and precise cell volume measurement by flow cytometry. *Cytometry Part A.* 1983;3(6):419-427.
76. Semevolos SA, Youngblood CD, Grissom SK, Gorman ME, Larson MK. Evaluation of two platelet-rich plasma processing methods and two platelet-activation techniques for use in llamas and alpacas. *American Journal of Veterinary Research.* 2016;77(11):1288-1294.
77. Nabity MB, Harr KE, Camus MS, Flatland B, Vap Linda M. ASVCP guidelines: Allowable total error hematology. *Veterinary Clinical Pathology.* 2018;47(1):9-21.
78. Stokol T, Erb HN. A comparison of platelet parameters in EDTA-and citrate-anticoagulated blood in dogs. *Veterinary Clinical Pathology.* 2007;36(2):148-154.
79. Rogausch H. Time-dependent reaction of human red cell deformability on sphering agents. *Pflügers Archiv.* 1976;362(2):121-126.

APPENDIX

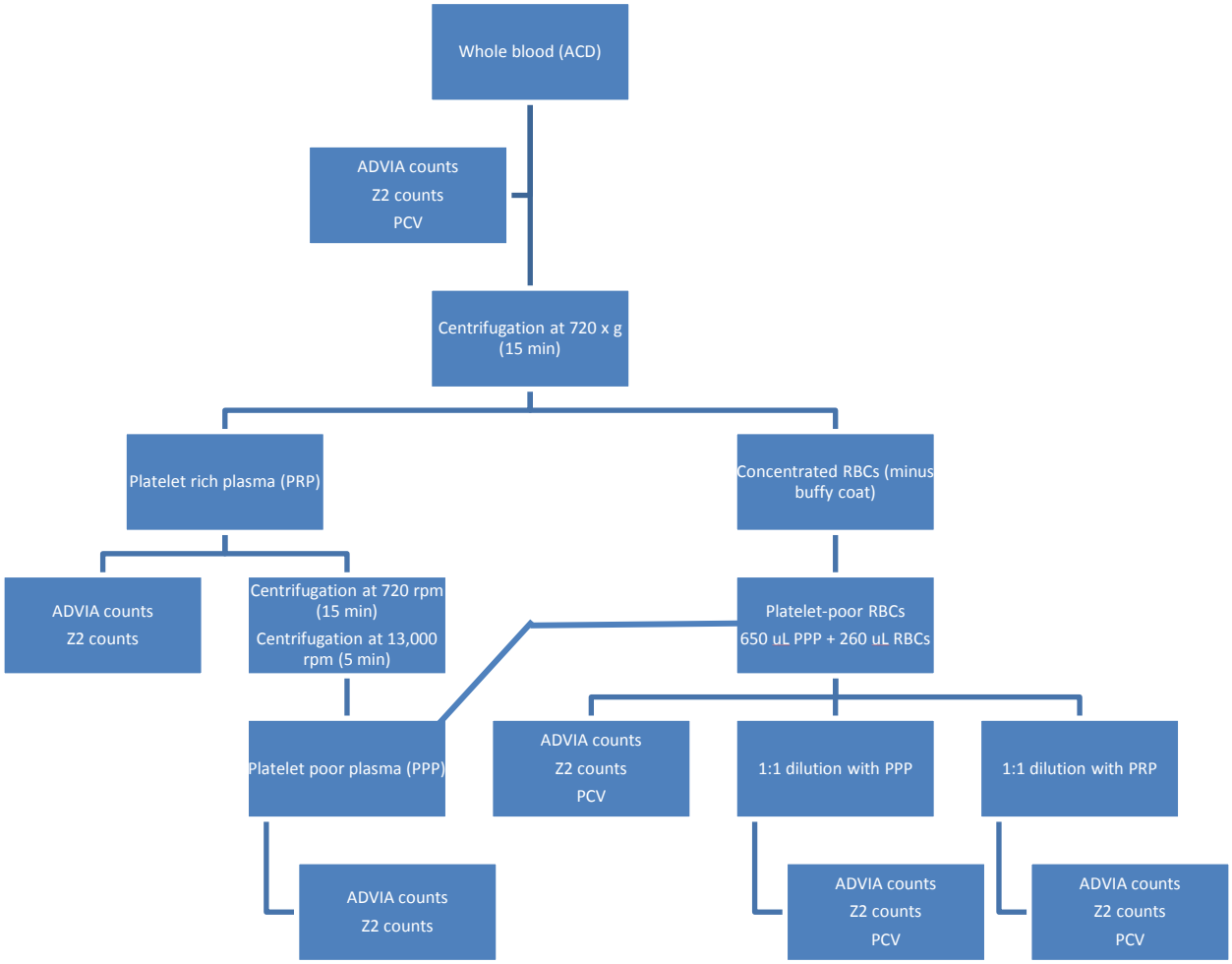

Figure 1. Methodology for analysis of whole blood, PRP, and RBCs

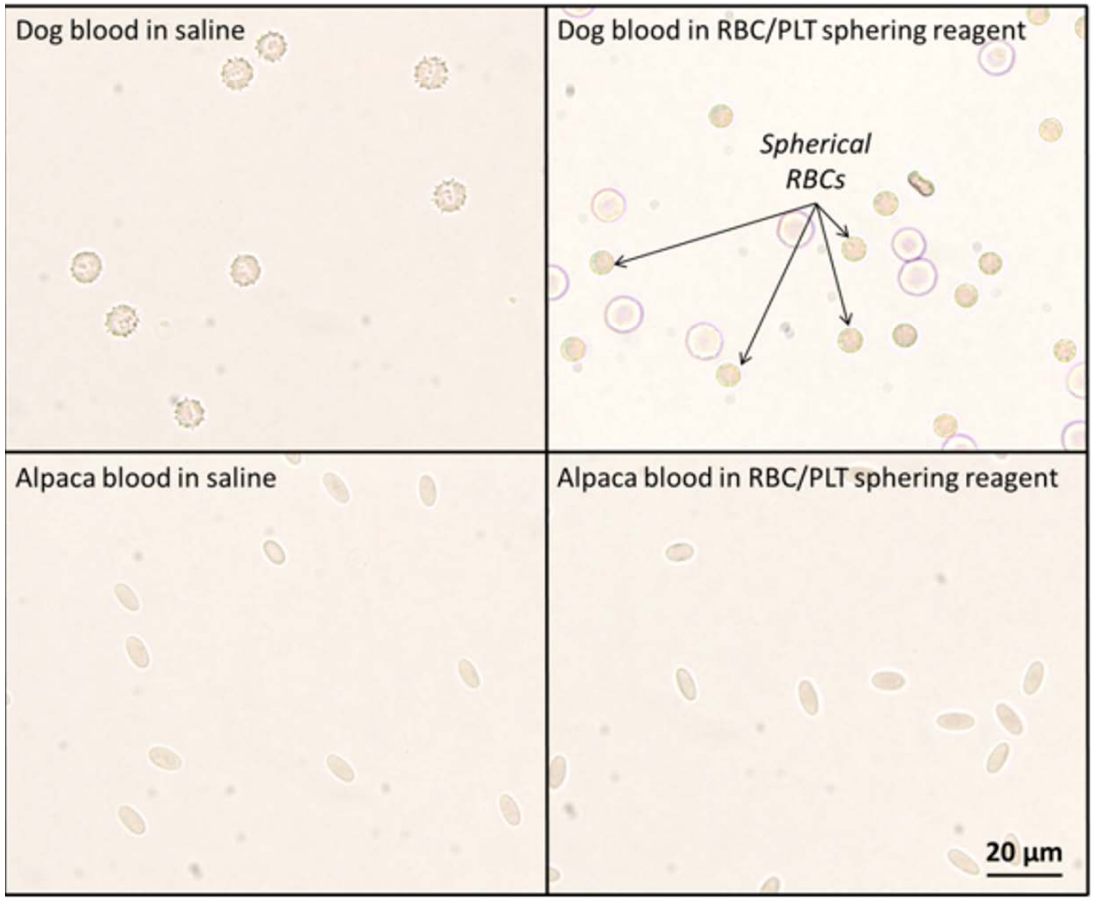

Figure 2. Light microscopic images of RBC sphering reactions

Table 1. RBC linearity data: measured and expected RBC counts

Dilution (plasma:RBC)	Average measured RBC count ($x10^6/\mu L$)	Expected RBC count ($x10^6/\mu L$)
Plasma	0.33	0
4:1	3.17	2.82
3:2	5.32	5.65
2:3	8.58	8.47
1:4	11.11	11.30
RBC	14.12	14.12

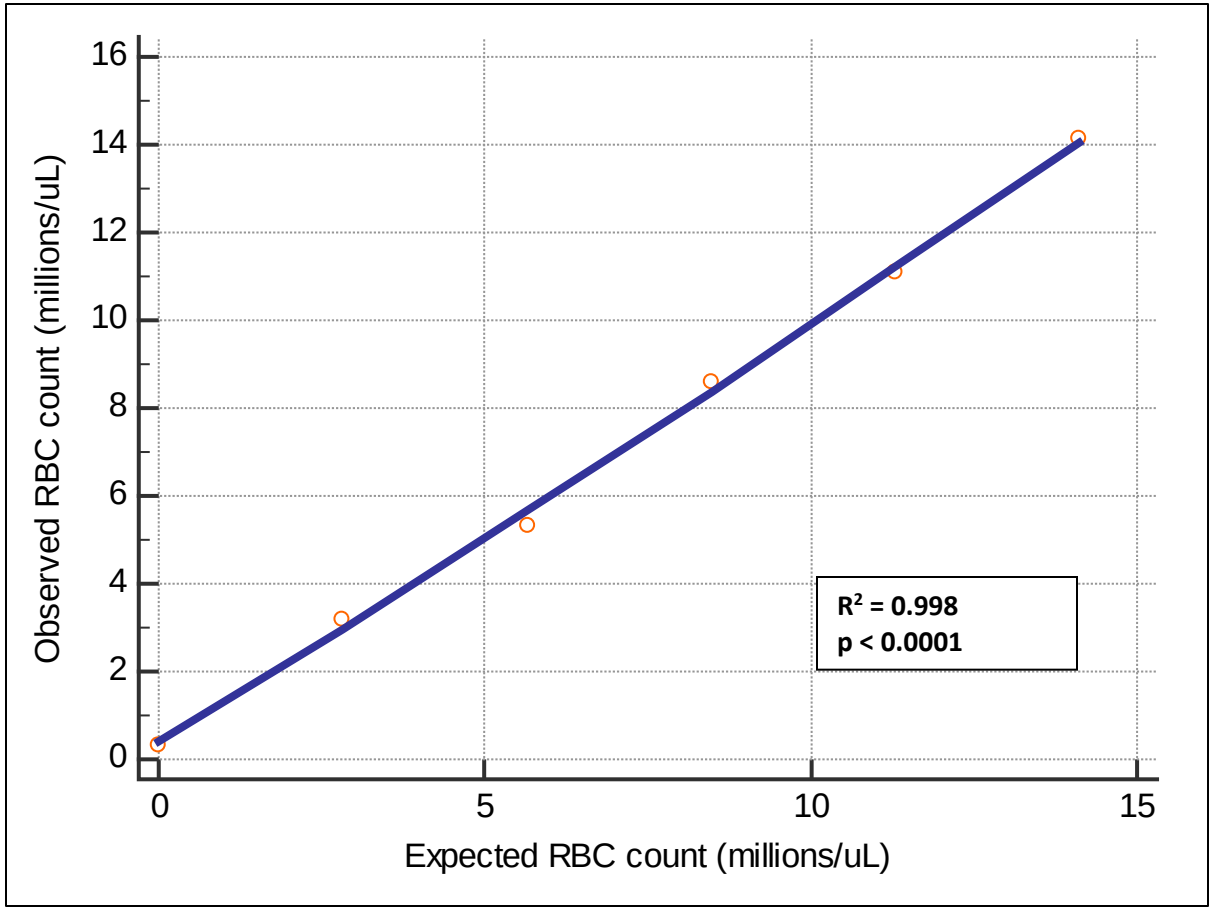

Figure 3. Correlation scatter plot with regression line for equine RBC dilutions

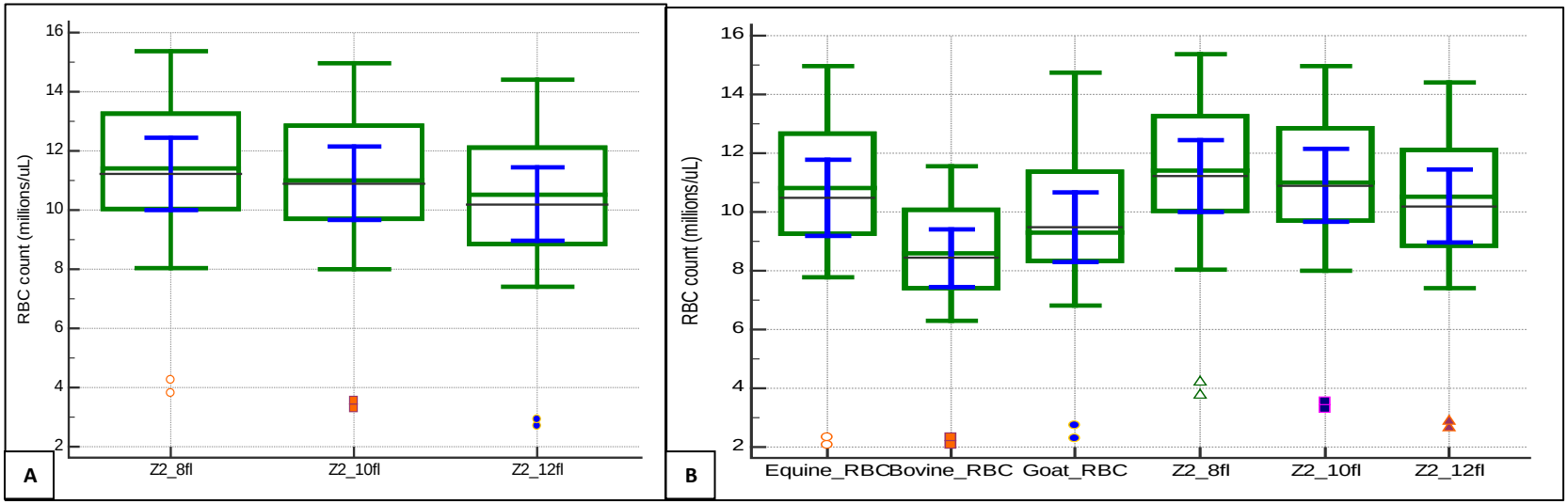

Figure 4. A) Comparison of mean (black lines) ± 95% CI (blue lines) for Z2 counts on camelid whole blood B) Comparison of mean ± 95% CI for Z2 counts and ADVIA RBC counts on camelid whole blood

Table 2. Summary of RBC and platelet counts for whole blood, platelet-poor RBCs, PRP, and PPP. NA=not applicable; ND=not determined.

	PCV (%)	ADVIA (equine)		Z2 particle count		
		RBC ($x10^6/\mu L$)	PLT ($x10^3/\mu L$)	8 fL	10 fL	12 fL
Whole blood					**($x10^6/\mu L$)**	
Llama 1	26	10.73	442	11.21	10.77	10.03
Llama 2	26	11.02	445	10.87	10.55	10.38
Llama 3	22	6.73	349	6.44	6.28	6.25
Llama 4	27	9.82	384	9.82	9.63	9.14
Platelet-poor RBCs					**($x10^6/\mu L$)**	
Llama 1	26	10.52	29	12.29	10.75	11.42
Llama 2	24	11.00	33	10.41	10.14	9.51
Llama 3	21	6.47	11	5.78	5.71	6.12
Llama 4	20	7.64	39	7.78	7.74	7.41
PRP					**($x10^3/\mu L$)**	
Llama 1	NA	0.06	486	125	75	35
Llama 2	NA	0.03	405	90	70	55
Llama 3	NA	0.02	243	95	35	25
Llama 4	NA	0.02	328	120	55	30
PPP					**($x10^3/\mu L$)**	
Llama 1	NA	0	ND			
Llama 2	NA	0	16			
Llama 3	NA	0	4			
Llama 4	NA	0	6			

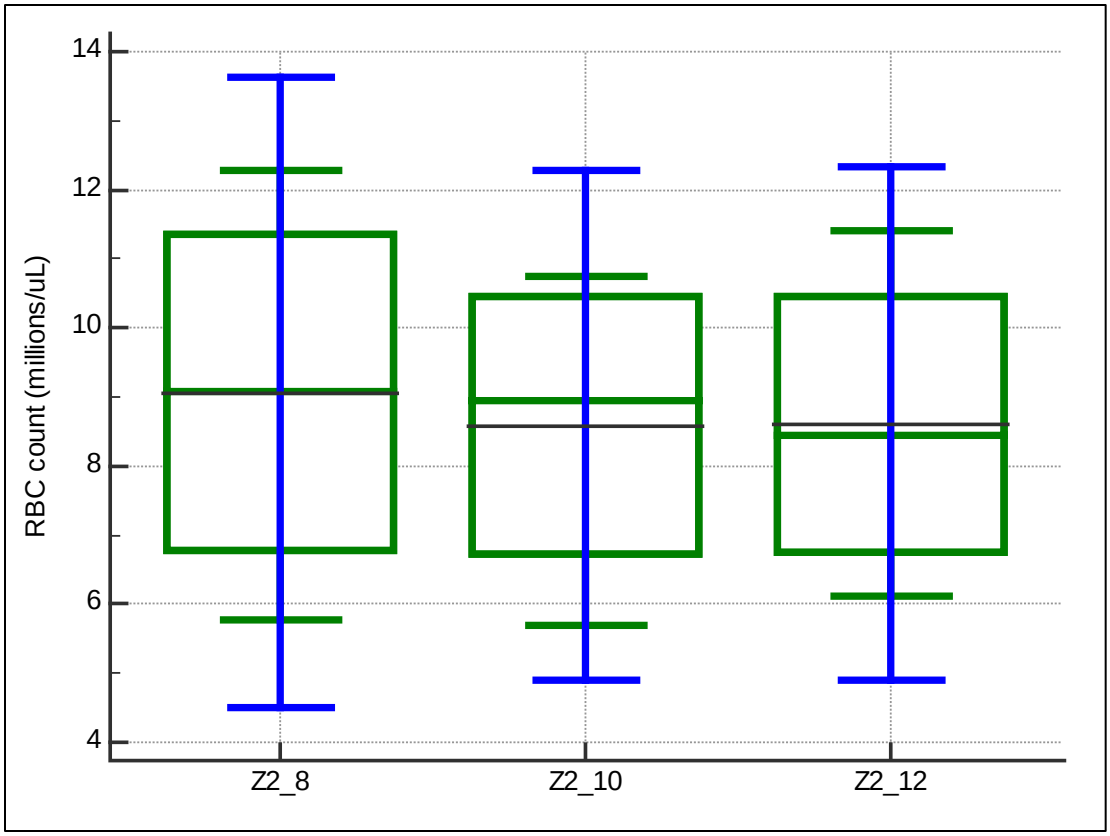

Figure 5. Comparison of mean (black line) ± 95% CI (blue lines) for Z2 counts on platelet-poor RBCs

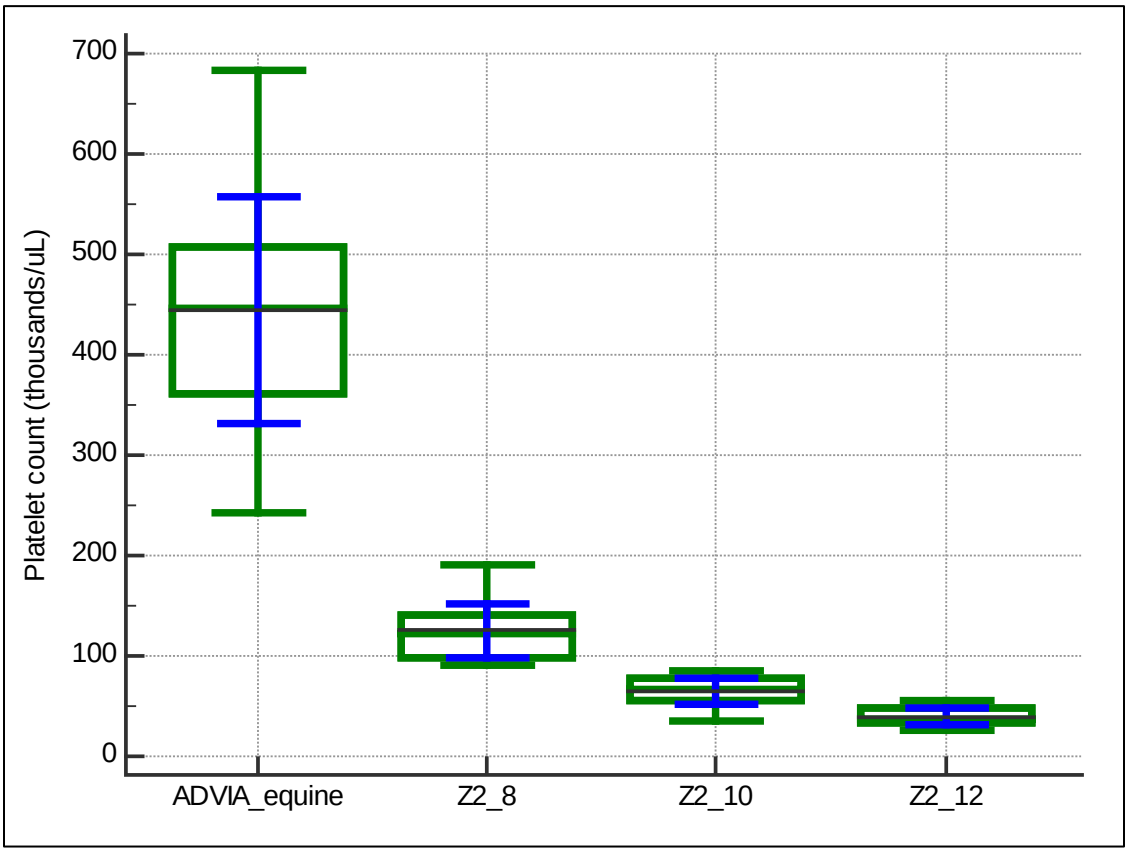

Figure 6. Comparison of mean (black line) ± 95% CI (blue lines) for ADVIA platelet counts (equine setting) and Z2 counts on PRP

Table 3. Method comparison summary of RBC count and MCV between ADVIA and Z2

					Passing-Bablok regression						Bland-Altman		
Analyte	**Test method**	**Comparative method**	**N**	**R**	**y-int**	**95% CI**	**Constant bias?**	**Slope**	**95% CI**	**Proportional bias?**	**Mean diff.**	**±1.96 SD**	**% of results within TE_a of comparative method**
RBC ($x10^6$/uL)	ADVIA equine	Z2 10 fL	25	0.96	-0.512	-1.631 to 0.468	No	1.01	0.922 to 1.11	No	-0.42	-1.47 to 0.64	88%
RBC ($x10^6$/uL)	ADVIA bovine	Z2 10 fL	25	0.92	-0.149	-0.856 to 0.973	No	0.785	0.687 to 0.873	Yes	-2.5	-4.3 to -0.7	0%
RBC ($x10^6$/uL	ADVIA goat	Z2 10 fL	25	0.94	-0.764	-1.92 to 0.338	No	0.943	0.840 to 1.07	No	-1.41	-2.88 to 0.06	28%
MCV (fL)	ADVIA equine	Calculated MCV from Z2 10 fL	25	0.65	-2.542	-11.99 to 5.314	No	1.426	1.10 to 1.828	Yes	6.8	1.3 to 12.3	4%
MCV (fL)	ADVIA bovine	Calculated MCV from Z2 10 fL	25	0.67	-3.01	-18.93 to 4.662	No	1.575	1.246 to 2.232	Yes	9.5	2.8 to 16.2	0%
MCV (fL)	ADVIA goat	Calculated MCV from Z2 10 fL	25	0.33	-34.11	-125.5 to 3.621	No	3.12	1.503 to 7.008	Yes	15.6	2.3 to 28.9	0%
MCV (fL)	Calc. from ADVIA equine	Calculated MCV from Z2 10 fL	25	0.70	-3.37	-13.34 to 1.718	No	1.181	0.972 to 1.596	No	1.7	-5.0 to 8.3	84%

Table 4. Method comparison summary of Hct and MCHC between automated and manual methods

					Passing-Bablok regression						Bland-Altman		
Analyte	**Test method**	**Comparative method**	**N**	**R**	**y-int**	**95% CI**	**Constant bias?**	**Slope**	**95% CI**	**Proportional bias?**	**Mean diff.**	**± 1.96 SD**	**% of results within TE_a of comparative method**
Hct (%)	ADVIA equine	PCV	156	0.89	-4.117	-6.375 to -2.107	Yes	1.378	1.316 to 1.450	Yes	6.5	-6.9 to 20.0	5%
Hct (%)	ADVIA bovine	PCV	155	0.94	-3.956	-6.30 to -1.80	Yes	1.222	1.150 to 1.30	Yes	3.1	-3.0 to 9.1	46%
Hct (%)	ADVIA goat	PCV	38	0.92	-10.60	-17.350 to -4.750	Yes	1.80	1.60 to 2.050	Yes	13.1	-4.2 to 30.5	5%
MCHC (g/dL)	ADVIA equine	Calculated MCHC from PCV	155	0.21	20.984	17.567 to 24.006	Yes	0.266	0.189 to 0.352	Yes	-7.6	-16.0 to 0.9	3%
MCHC (g/dL)	ADVIA bovine	Calculated MCHC from PCV	155	-0.03	-6.604	-27.716 to 6.433	No	1.081	0.752 to 1.611	No	-3.1	-11.8 to 5.6	48%
MCHC (g/dL)	ADVIA goat	Calculated MCHC from PCV	38	0.39	-49.013	-109.595 to -15.474	Yes	1.887	1.069 to 3.377	Yes	-11.6	-24.2 to 1.2	0%

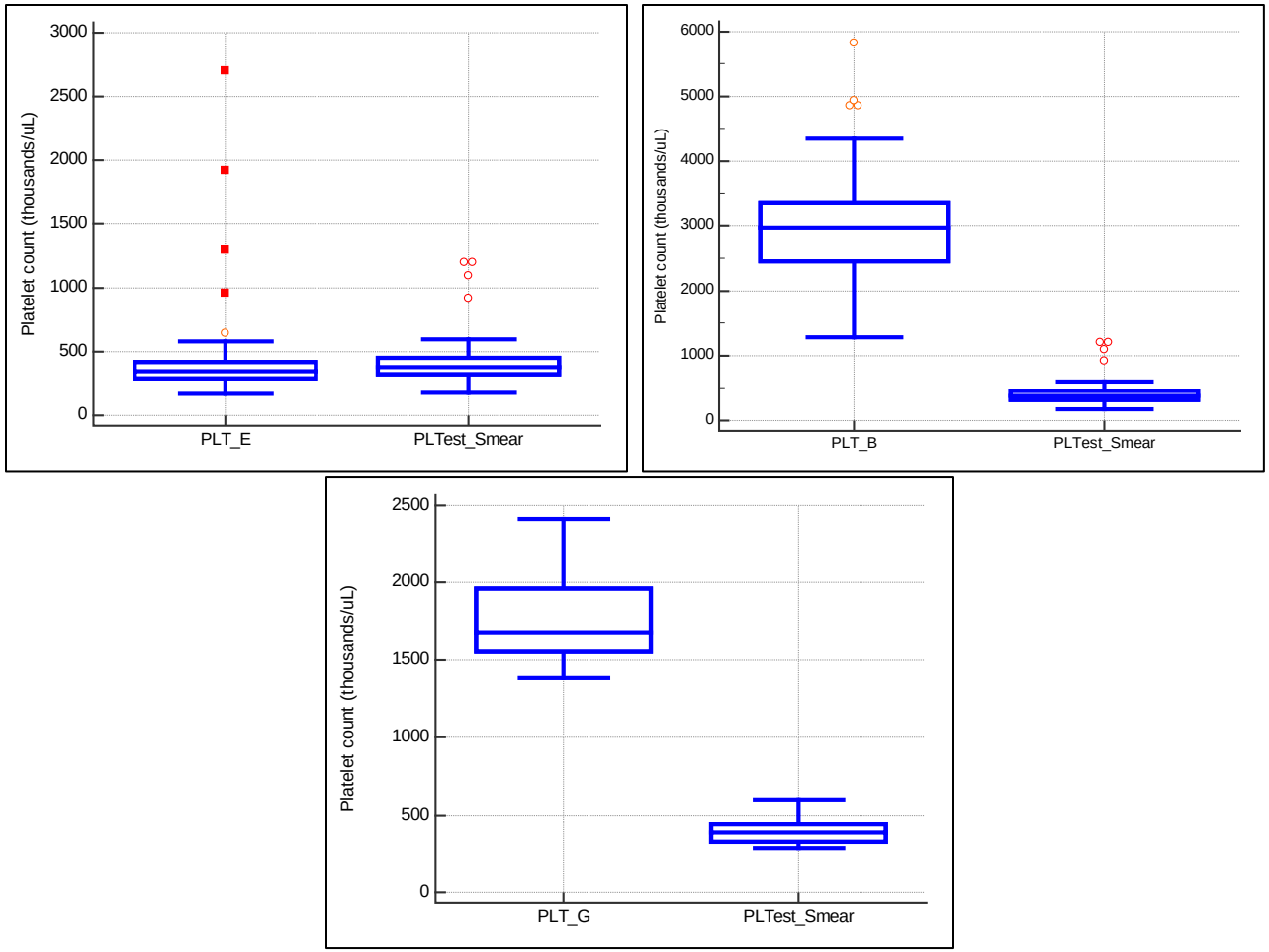

Figure 7. Comparison of ADVIA platelet counts with a manual platelet estimate

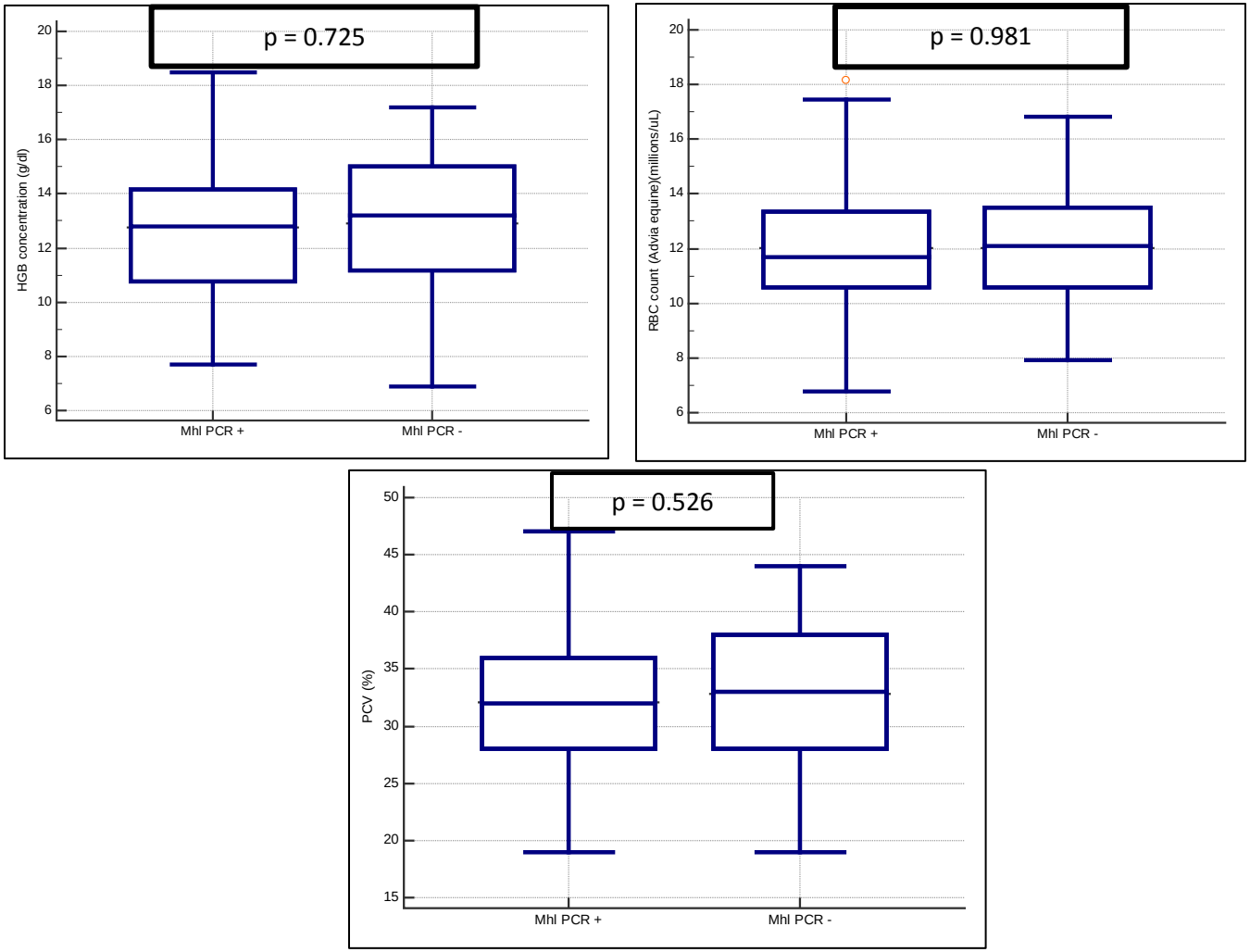

Figure 8. Comparison of RBC values between Mhl-positive and Mhl-negative camelids

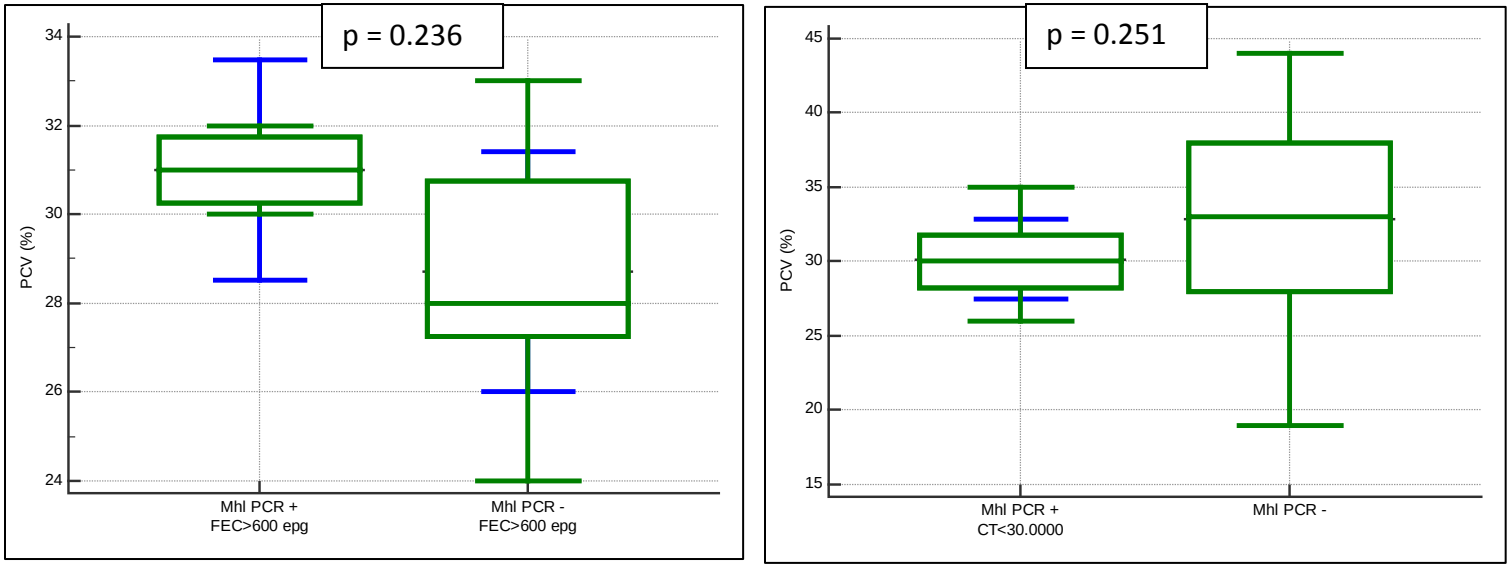

Figure 9. Comparison of spun PCV A) between Mhl-positive and Mhl-negative animals with FEC>600 epg, and B) between Mhl-positive animals with CT values<30.0 and Mhl-negative animals

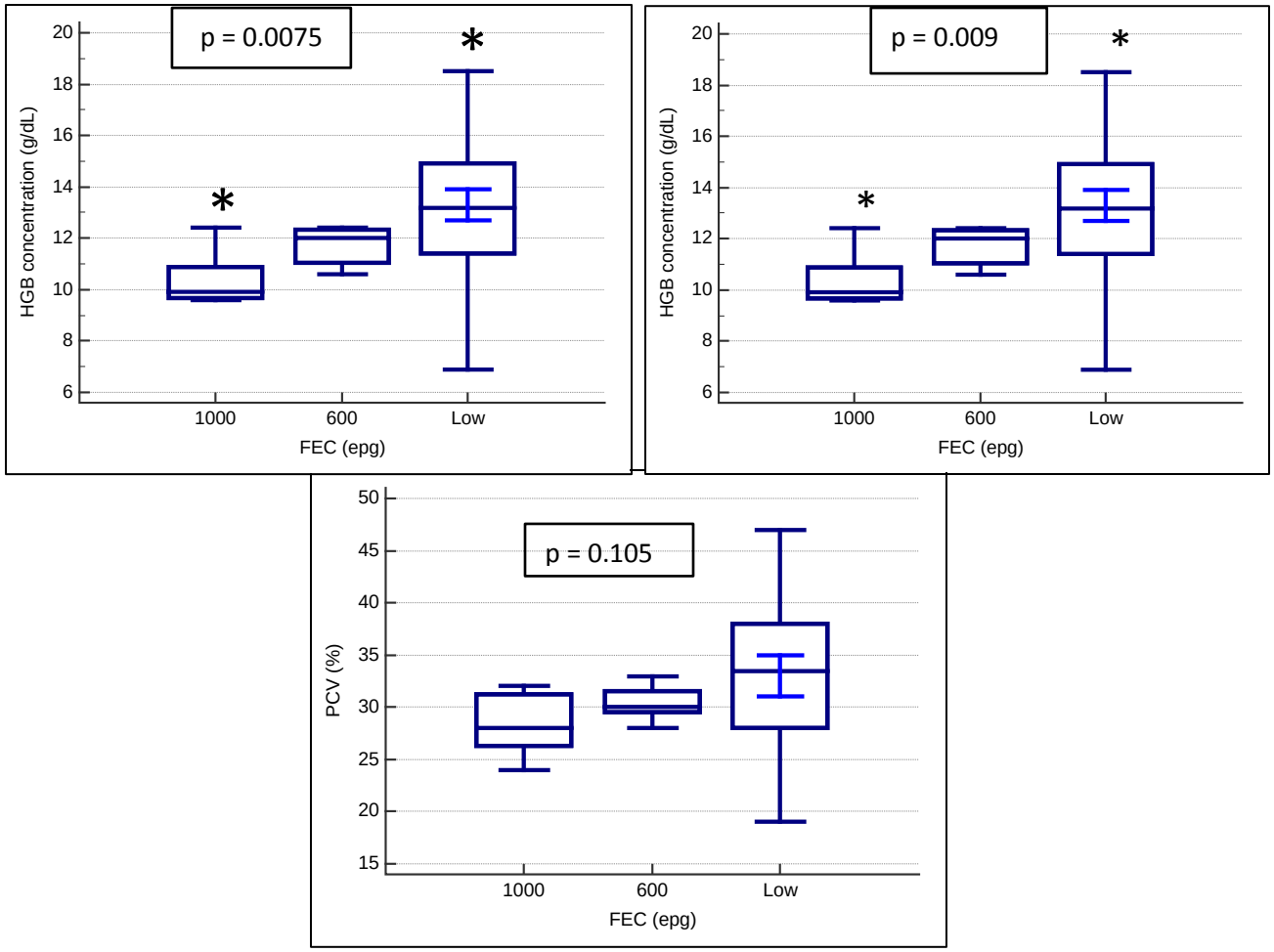

Figure 10. Comparison of RBC values between FEC strata (low = <450 epg; 600 = 600<FEC<1000; 1000 = ≥1000 epg)

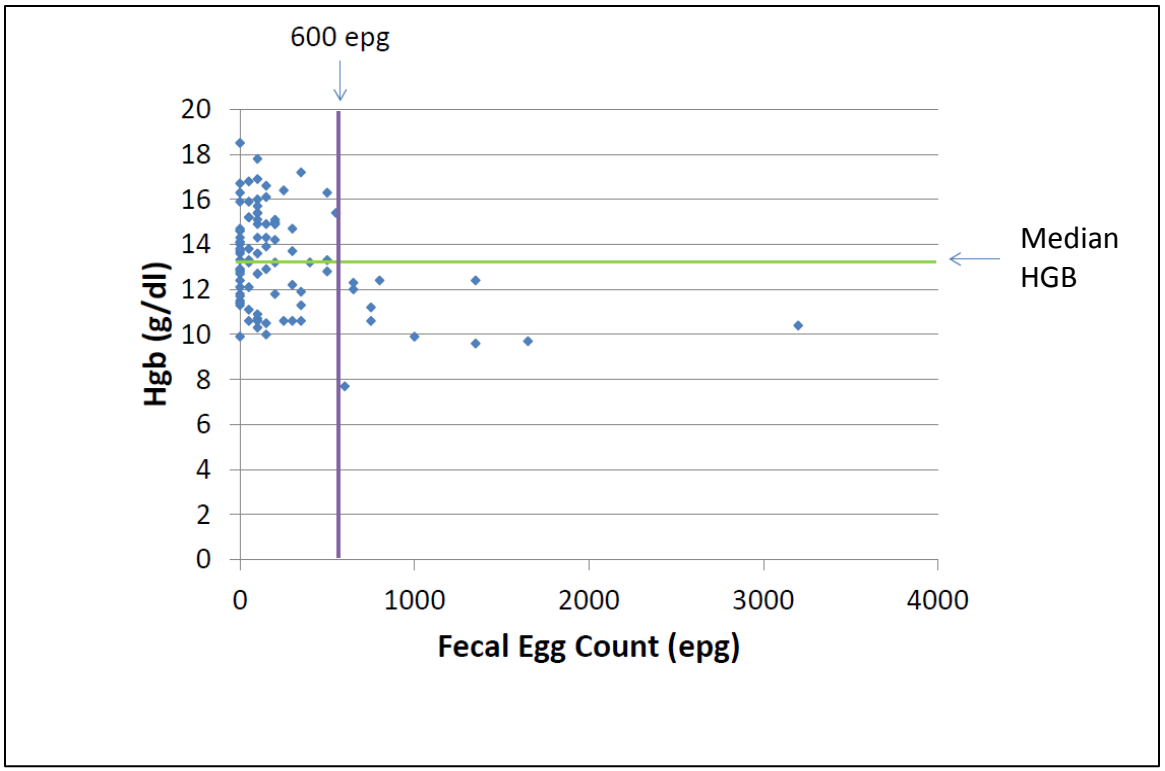

Figure 11. Distribution of hemoglobin concentration by FEC

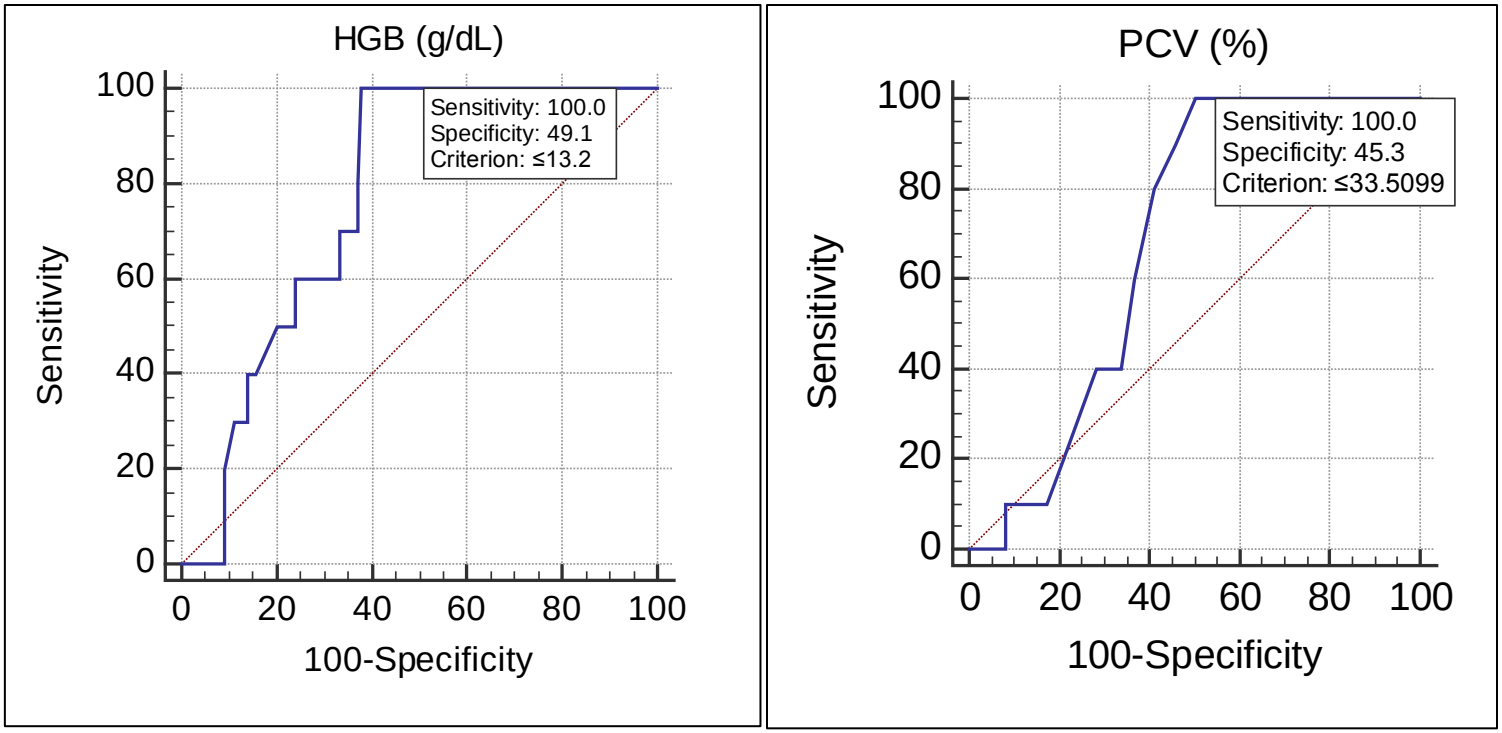

Figure 12. ROC curve analysis for HGB and spun PCV, using an FEC threshold of 600 epg

VITA

Lisa C. Viesselmann was born and raised in Wisconsin. She attended the University of Wisconsin-Madison, where she received a Bachelor of Science degree in Biology in 2006, and a Doctor of Veterinary Medicine degree in 2010. Upon graduation from veterinary medical school, she was commissioned as a Captain in the United States Army Veterinary Corps, where she served for 5 years. After separation from active duty in 2015, she started a clinical pathology residency at the University of Tennessee - College of Veterinary Medicine. She began a Master of Science degree in the Comparative and Experimental Medicine program at UT in 2016, and plans to graduate in May 2018. She will also complete the clinical pathology residency program in June 2018, and hopes to continue working in academia.